AWARD NUMBER: W81XWH-14-1-0086

TITLE: Targeting the S1P Axis and Development of a Novel Therapy for Obesity-Related Triple-Negative Breast Cancer

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REPORT DATE: September 2016

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2016	Annual	1 Sep 2015 - 31Aug2016

4. TITLE AND SUBTITLE
Targeting the S1P Axis and Development of a Novel Therapy for Obesity-Related Triple-Negative Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0086

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Virginia Commonwealth University School of Medicine
Richmond, VA 23284

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The majority of breast tumors express the estrogen receptor α (ERα), which plays important roles in breast cancer pathogenesis and progression, and hormonal therapies, such as tamoxifen, are the first line of adjuvant therapy (1, 2). Unfortunately, half of these patients will ultimately fail therapy due to de novo or acquired resistance. Moreover, patients with ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2) triple negative breast cancer (TNBC), which is aggressive with high recurrence, metastatic, and mortality rates (3), do not respond to hormonal therapies and have limited treatment options. Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis (4), which may be due in part to the high frequency of TNBC and ineffectual hormonal therapy (5). However, the links between obesity and breast cancer are not understood and is the focuse of our study. As hormonal therapy is so effective with relatively few side effects, the possibility of reversing hormonal unresponsiveness is an appealing treatment approach. Our study will lead to novel therapies that will overcome the overarching challenges of developing safe and effective drugs for treating obesity-promoted cancers and TNBC and will identify the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), produced by sphingosine kinases (SphK1 and SphK2), as a critical factor that links obesity and chronic inflammation to drive breast cancer growth and metastasis.

15. SUBJECT TERMS-

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	44	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Breast cancer remains the most common malignant disease in women. The majority of breast tumors express the 66 kDa estrogen receptor α (ERα66), which plays important roles in breast cancer pathogenesis and progression, and hormonal therapies, such as tamoxifen, are the first line of adjuvant therapy (1, 2). Unfortunately, half of these patients will ultimately fail therapy due to *de novo* or acquired resistance. Moreover, patients with ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2) triple negative breast cancer (TNBC), which is aggressive with high recurrence, metastatic, and mortality rates (3), express the 36 kDa splice variant ER-α36, do not respond to hormonal therapies and have limited treatment options. Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis (4), which may be due in part to the high frequency of TNBC and ineffectual hormonal therapy (5). However, the links between obesity and TNBC are not understood and is the focus of our study. As hormonal therapy is so effective with relatively few side effects, the possibility of reversing hormonal unresponsiveness is an appealing treatment approach. We hope that our study will lead to novel therapies that will overcome the overarching challenges of developing safe and effective drugs for treating obesity-promoted cancers and TNBC and will identify the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), produced by sphingosine kinases (SphK1 and SphK2), as a critical factor that links obesity and chronic inflammation to drive breast cancer growth and metastasis.

2. KEY WORDS

sphingosine-1-phosphate, sphingosine kinase, FTY720 (fingolimod, Gilenya), triple negative breast cancer, ERα, obesity, histone deacetylase, inflammation, tamoxifen resistance

3. ACCOMPLISHMENTS

3.1. Major Goals of the Project

Our project has three major aims.

Aim 1. Determine the role of SphK1 and S1P in obesity promoted chronic inflammation and tumor progression and decipher the molecular links between the SphK1-S1P-S1PR1 axis and persistent NF-kB and STAT3 activation.

Aim 2. Target the SphKs/S1P/S1PR1 axis with fingolimod/FTY720 for treatment of obesity associated breast cancer to suppress the malicious amplification cascade, and reactivate ER expression in ER-negative breast cancer.

Aim 3. Examine the association of the SphKs/S1P/S1PR1 axis in human breast cancer and prognosis.

The inability to effectively predict, prevent, and treat metastatic breast cancer is a major problem in breast cancer care. This proposal provides evidence that the SphKs-S1P-S1PR1 axis is one of the critical factors that bridges obesity, chronic inflammation, and breast cancer and paves the way for development of new adjuvant therapies targeting this axis as a promising strategy for effective treatment of advanced and refractory breast cancer.

3.2. Accomplishments Under These Goals and Significant Results

Aim 1. Role of SphK1 And S1P In Obesity Promoted Breast Cancer Progression

Modified Breast Cancer Model for Preclinical Immunotherapy Research

Immunotherapy for breast cancer is now receiving much attention. Applicable animal models that more accurately mimic human breast cancer are needed for preclinical research. In a recent collaborative study with Dr. Takabe's group, we developed an improved method for orthotopic inoculation of syngeneic breast cancer cells as a suitable preclinical animal model for

investigation of immunotherapy that should be useful in evaluating the efficacy of tumor regression mediated by immune responses (6).

Levels of S1P in Mammary Tumor Interstitial Fluid Correlate with Tumor Growth

We completed our studies investigating the involvement of SphK1 and S1P in high fat diet promoted breast cancer progression and metastasis. Increased S1P levels were detected in the primary and metastatic tumors. However, despite its critical roles, the levels of S1P in interstitial fluid (IF), an important component of the tumor microenvironment, have not previously been measured due to a lack of efficient methods for collecting and quantifying IF. Therefore, we recently developed such methods (7). Although as expected, we found that levels of S1P in normal mammary glands are relatively low, much lower than those of sphingosine; surprisingly however, high concentrations of bioactive sphingolipids (reaching 0.6 μM S1P and 0.2 μM dihydro-S1P) were observed in IF from normal mammary glands. These sphingolipid metabolites in mammary gland IF from SphK1 null mice were decreased, supporting the notion that SphK1 plays a pivotal role in regulating levels of these metabolites in IF from mammary glands. In contrast, higher levels of S1P were found in mammary gland IF from SphK2 knockout mice (Fig. 1A). We are determining the role of host SphK1 and SphK2.

We previously showed in a syngeneic mouse breast cancer model that when 4T1-luc2 murine mammary cancer cells were orthotopically implanted into the chest mammary fat pad of these immunocompetent mice that S1P levels were increased in tumors and correlated with tumor growth (8). Thus, we next examined levels of the sphingolipid metabolites in IF from these tumors. S1P and dihydro-S1P were increased by more than 3-5 fold in the tumor IF than in IF from normal breast tissue (compare Fig. 1A to 1B). Because we have reported that oral administration of FTY720/fingolimod reduces tumorigenesis (9), and because of the known effects of FTY720 on S1P signaling, we also treated 4T1 tumor bearing mice with FTY720 and examined correlations between tumor burden and levels of bioactive sphingolipids in tumor IF. FTY720 greatly reduced tumor growth, as demonstrated by in vivo bioluminescence and tumor volume measurements, and levels of S1P and dihydro-S1P in tumor IF were also significantly decreased compared to saline treated animals (Fig. 1B). While these observations further support our proposal that S1P may have an important role within the tumor microenvironment, they also provide an important insight into the possible mechanisms of action of FTY720 on cancer progression due to reduction of S1P via suppression of SphK1.

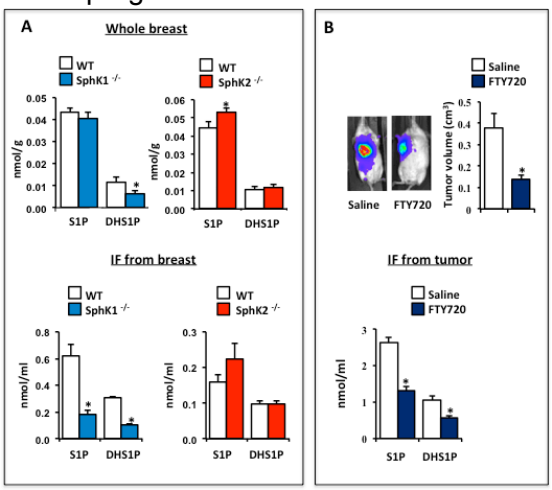

Figure 1. (A) Levels of S1P in mammary gland IF. S1P and dihydro-S1P (DHS1P) in mammary glands, and in IF from the mammary glands of SphK1$^{-/-}$ mice, SphK2$^{-/-}$ mice and their WT littermates were determined by LC-ESI-MS/MS. **(B) Levels of S1P in breast tumor IF correlate with tumor growth**. 4T1-luc2 cells were surgically implanted in mammary glands. Tumor-bearing mice were randomized into 2 groups and treated daily by gavage with saline or FTY720 (1 mg/kg). IVIS images of tumors on day 14 and tumor volume determined. Levels of S1P and DHS1P in breast tumor IF were determined by LC-ESI-MS/MS. Data are mean ± SEM. *, P < 0.05.

High Fat Diet and S1P Produced by SphK1 Link Increased Metastatic Niches and Lung Metastasis

We reported last year that S1P produced by SphK1 plays a critical role in obesity-promoted inflammation and tumor progression in both syngeneic (orthotopic implantation of

E0771 cells) and genetically engineered (MMTV-PyMT) (9) breast cancer mouse models. In these mice, after HFD feeding, we also found increased expression of SphK1 and S1PR1 as well as increased levels of the key inflammatory cytokines, IL-6 and TNF-α, in tumors. Therefore, we next examined whether S1P secreted from the primary tumor into the tumor IF, which drains into systemic circulation via lymphatic flow, due to HFD feeding could also promote formation of "metastatic niches" that assist circulating cancer cells to form lung metastases (Fig. 2A). We found that tumor conditioned medium (TCM) from E0771 cells overexpressing SphK1 (TCM-*Sphk1*^{high}) enhanced formation of metastatic niches in the lung compared to TCM derived from control tumor cells. Importantly, feeding HFD for 12 weeks prior to treating mice with TCM-*Sphk1*^{high} induced significantly more metastatic nodules, which was accompanied by extensive metastasis at day 7 post-tumor cell tail vein injections compared to mice on normal diet (Fig. 2B,C). HFD-induced obesity also increased S1P-mediated signaling including pERK, pAKT, pStat3, and pp65 in these lungs (Fig. 2D). Treatment of the mice with clinically relevant doses of FTY720 greatly reduced HFD-induced formation of metastatic niches and suppressed S1P-mediated signaling pathways (Fig. 2).

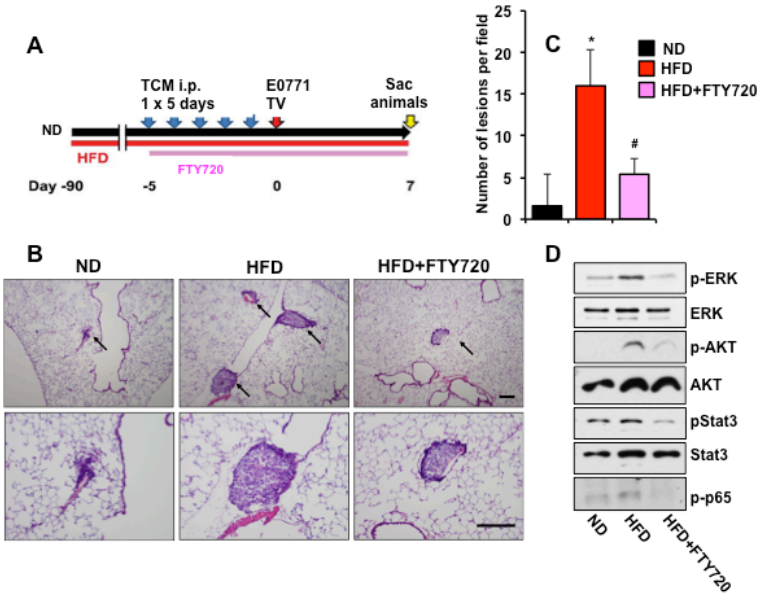

Figure 2. HFD increases metastatic niches in the lung that are suppressed by FTY720 treatment. (A) Scheme for determination of metastatic niche formation in the lungs of HFD fed mice utilizing E0771 cell conditioned media (TCM). (B-D) Mice were fed with HFD or normal diet (ND) for 12 weeks and then treated with TCM-*Sphk1*^{high} for 5 days prior to tail vein injections of E0771 cells. Mice were treated daily with FTY720 (1 mg/kg/day) or PBS by gavage. (B) Lungs were stained with H&E and metastatic lesions indicated by arrows. (C) Number of metastatic lesions are expressed as means ± SEM. * P<0.05 vs. ND; $^{\#}$ P<0.05 vs HFD. (D) Equal amounts of lung lysates were analyzed by western blotting with the indicated antibodies.

<u>Aim 2.</u> Targeting the SphKs/S1P/S1PR1 axis with fingolimod/FTY720 for treatment of obesity associated breast cancer.

FTY720 Attenuates Obesity-Induced Lung Metastasis

In agreement with our previous study in MMTV-PyMT transgenic mice (9) (Fig. 3A), histological analysis of the lungs from mice bearing E0771 tumors showed that HFD increased metastatic lesions compared to mice fed normal diet (Fig. 3B). Therefore, we next tested the effect of FTY720 on obesity-related cancer metastasis in both the MMTV-PyMT spontaneous breast cancer model as well as in the E0771 syngeneic orthotopic implantation model where cancer cells metastasize from the breast to the lung, that more closely mimics the pathology of human breast cancer metastasis. FTY720 administration significantly reduced the lung metastasis in these mice (Fig. 3A,B). Taken together, our results suggest a critical role for S1P produced by SphK1 in obesity-related inflammation, metastatic niche formation and breast cancer metastasis. Our findings also indicate that "inside-out signaling" by S1P could be a

promising target for treatment of breast cancer metastasis in the setting of obesity-related inflammation. Considering the fact that the population of obese breast cancer patients is large in the US, S1P targeted therapy for metastatic breast cancer may be expected to have a significant impact on the overall health of our country.

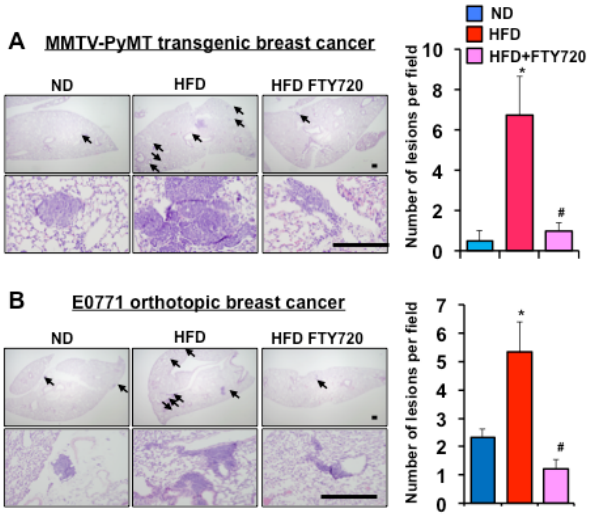

Figure 3. FTY720 suppresses lung metastasis in high fat diet fed MMTV-PyMT transgenic and E0771 syngeneic orthotopic breast cancer mice. (A) MMTV-PyMT transgenic mice were fed with normal diet (ND), HFD with saline (HFD), or HFD with FTY720 (1 mg/kg/day) (HFD+FTY) by gavage for 10 weeks. (**B**) C57Bl/6 mice were fed with ND or HFD for 12 weeks, before E0771 cells were implanted into the chest mammary fat pad under direct vision. When tumor sizes reached 5 mm in diameter, mice with HFD were treated with FTY720 (HFD+FTY) or saline (HFD) for 18 days. Lungs were stained with H&E and metastatic lesions indicated by arrows. Number of metastatic lesions was determined and expressed as mean ± SEM. * P<0.05 vs. ND; # P<0.05 vs. HFD.

<u>Aim 3.</u> Examine the association of the SphKs/S1P/S1PR1 axis in human breast cancer and prognosis.

S1P Levels in Interstitial Fluid from Patient Breast Cancer Tissue are Higher than those from Normal Breast Tissue

We have begun examining the association of the SphK1/S1P axis with breast cancer disease progression and obesity. As SphK1 is activated by phosphorylation in breast cancer cells, we are examining the activation status of SphK1 in breast cancer patients by immunostaining of a tissue microarray with a phospho-specific SphK1 antibody. This study has not yet been completed but it seems that activated SphK1 is more prevalent and increased in larger tumors (higher T stage) and particularly in tumors from patients with lymph node metastases (higher TNM stage). We also examined the levels of sphingolipid metabolites in IF from human patients with breast cancer. To this end, we compared IF from breast tumor tissue and normal breast tissue from two different areas (peri-tumoral areas and distant areas from the tumor) in each patient with breast cancer and determined levels of sphingolipids in the fluid by the state of the art mass spectrometry method we developed. We found that sphingosine and S1P levels, were significantly higher in the breast tumor tissue IF than in the normal breast tissue IF, but not dihydro-S1P (probably due to extremely low levels) (Fig. 4).

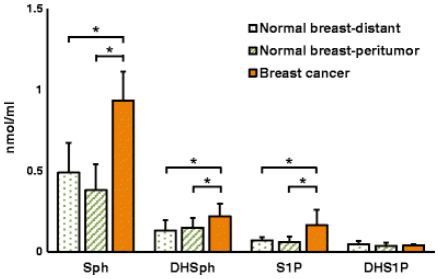

Figure 4. Levels of S1P in interstitial fluid from breast tumor and normal breast tissue of patients with breast cancer. IF was obtained from breast cancer tissue and normal breast tissue obtained from areas distant from tumor and peritumoral areas. Levels of sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in the IF from tumor and normal breast tissue were determined by LC-ESI-MS/MS. Data are mean ± SEM. *, P<0.05

There were no significant differences in levels of sphingosine, dihydrosphingosine, S1P or dihydro-S1P between IF from normal breast tissue that is distant from tumor and that from peri-tumor normal breast tissue (Fig. 4). Our findings

suggest that S1P secreted from tumor cells to IF may be important for metastasis by stimulating S1P signaling critical for cancer progression and highlights its important role in the tumor microenvironment. This is the first report of the measurement of S1P in tumor IF from human patients with breast cancer.

Estrogen Receptor Alpha Splice Variant Erα36 Regulates SphK1/S1P Axis In Triple Negative Breast Cancer

It is very well known that estrogen receptor-α (ERα) and its ligand 17β-estradiol (E2) play critical roles in breast cancer. It is also well accepted that E2 elicits genomic effects in ERα-positive breast cancers that are important for tumor growth by binding to ERα66 and ERα46. Triple negative breast cancers (TNBC) lack ERα66 and thus do not respond to hormonal therapy with tamoxifen. Nevertheless, TNBC cells express the ERα36 splice variant on the plasma membrane that elicits rapid, non-genomic responses to E2 and ERα36 has been implicated in tamoxifen resistance. We previously found that E2 activates SphK1, producing S1P that in turn stimulates S1P receptors, important for cell growth and survival, motility, and invasiveness of breast cancer cells (10). However, the E2 receptor involved in SphK1 activation and formation of S1P unexpectedly made an important observation. Utilizing TNBC cells that only express ERα36, we found that treatment with E2 rapidly increased intracellular and secreted S1P measured by LC-ESI-MS/MS. Since this was an intriguing finding, we pursued it further and observed that even membrane-impermeable E2-BSA increased S1P secretion (Fig 5A). Immunoblotting lysates from these TNBC cells with a SphK1 phospho-specific antibody showed that both E2 and E2-BSA activated SphK1 (Fig. 5B). Confocal microscopy also revealed that E2 induced translocation of SphK1 to the plasma membrane in TNBC cells that only express ERα36. Furthermore, we found that both an ERα36 neutralizing antibody and downregulation of ERα36 suppressed E2-induced SphK1 activation and S1P production and secretion (Fig. 5A,B). These data demonstrate that ERα36 is the E2 membrane receptor required for E2-mediated SphK1 activation and rapid secretion of S1P, which regulates some of the non-genomic effects of E2. All together, our data suggests that targeting the SphK1/S1P axis may potentially be a new therapeutic option for treatment of TNBC.

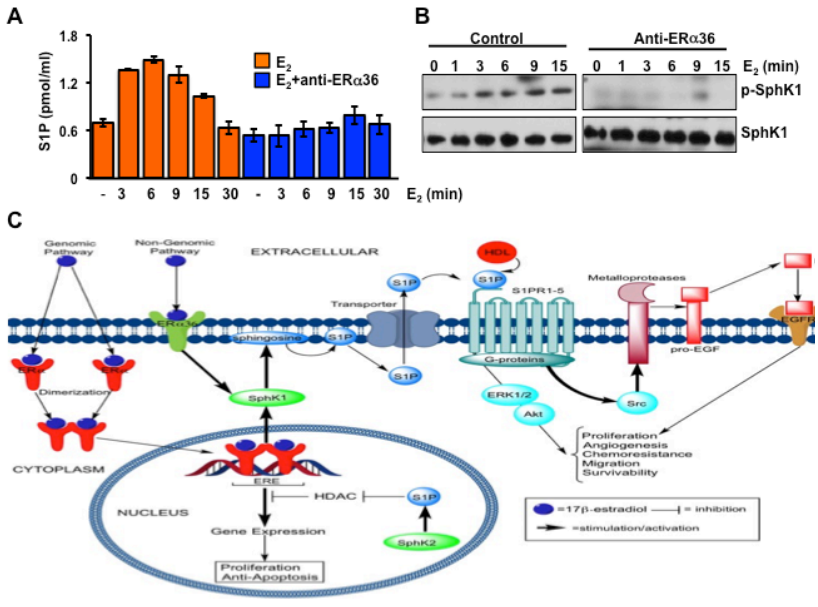

Figure 5. E2 stimulates S1P through ERα36 in TNBC. (A,B) MDA-MB-231 breast cancer cells were pre-treated with or without the blocking antibody, Anti-ERα36 (1:500), for 30 min and then treated with E2 (10^{-7} M) for the indicated times. (A) Levels of secreted S1P were determined by LC-ESI-MS/MS. * $p < 0.05$ compared to controls. (B) Cellular proteins were separated by SDS-PAGE and immuno-blotted with antibodies against p-SphK1 and SphK1. (C) Scheme illustrating the key role of SphK1 and S1P in non-genomic effects mediated by binding of E2 to ERα36. Modified from (11).

We are very excited about this finding as it not only provides for the first time basic

understanding of the link between ER and S1P, but also has important clinical implications. ERα36 is an important cause of tamoxifen resistance as tamoxifen, which is a ligand for this receptor, upregulates its expression and downregulates ERα66. Our findings suggest a previously unrecognized key player in this malicious link, namely activation of SphK1 and production of S1P by binding of tamoxifen to ERα36. S1P in turn transactivates the EGFR (Fig. 5C), switching growth status from estrogen-dependent to growth factor-dependent and thus leads to tamoxifen resistance. Further studies are needed to substantiate this discovery and examine the key role of the SphK1/S1P axis in tamoxifen resistance.

3.3. Opportunities for Training and Professional Development

Although the project was not designed to provide training and professional development opportunities, we should point out that the VCU School of Medicine developed several new programs for enhancing training and professional development of graduate students and postdoctoral fellows in recognition of the important roles they fulfill. This provides them with career and mentoring resources including FASEB Individual Development Plan, Individual Development Plan web-based tool, job opportunities in BioCareers, career resources from AAAS, CV/resume writing and samples from UCSF Office of Career and Professional Development, career development websites. For graduate students, these functions reside within the Office of Graduate Education. While no graduate students were included in the original proposal, Melissa Maczis, who rotated in my lab during her first year as a PhD student, decided to join my lab two years ago and was supported by the VCU School of Medicine for the first two years. I have already begun advising her on career development. Using the "my Individual Development" plan website, she created an Individual Development Plan (IDP) she is using to record the immediate and long term objectives of her research and plan of her career path. She has been making outstanding progress toward accomplishing her career goals.

3.4. How were the results disseminated to communities of interest

We presented several research lectures on this project to the cancer research community at the Massey Cancer Center Retreat and in the regular meetings of the Massey Cancer Center Cancer Cell Signaling Program, which I direct together with Dr. Andrew Larner. We also presented this work to the international scientific community (See below).

Dr. Spiegel presented a Keynote Address as a JLR Special Lecture: Sphingosine1phosphate from Bench to Clinic: Evolving concepts. FASEB Science Research Conference - Lysophospholipids and related mediators - From bench to clinic. Banff, Alberta, Canada. August 23-28, 2015

Dr. Spiegel presented: Sphingosine-1-phosphate and estrogen signaling in breast cancer. 56th ABR Symposium, Bologna, Italy. October 5-6, 2015.

Dr. Spiegel presented: Role of the sphingosine-1-phosphate axis in the tumor microenvironment and development of a novel therapy for obesity-related triple-negative breast cancer. Frontiers in Basic Cancer Research Conference, Philadelphia, PA. October 23-26, 2015

Dr. Spiegel presented: The road from Wilchek to sphingosine-1-phosphate. Affinity and Biorecognition. Meir Wilchek's 80th Birthday Symposium. Weizmann Institute of Science, Rehovot, Israel. October 27-30, 2015

Dr. Spiegel presented: The key role of sphingosine-1-phosphate in the link between inflammation and cancer. The 49th Annual Miami Winter Symposium on Inflammation. Miami, FL. January 24-27, 2016

Dr. Spiegel presented: Sphingosine-1-phosphate: A Bridge from Bench to Clinic. ASBMB 2016

Annual Meeting, San Diego, CA. April 2-6, 2016

Dr. Spiegel presented: Sphingosine-1-phosphate: From bench to Translational Medicine. Lipid Mediators In Health and Disease II, La Jolla, CA. May 19-20, 2016

Dr. Spiegel present an invited Keynote Address: Sphingosine-1-phosphate at the crossroads between cancer and inflammation. FASEB Summer Research Conference, Phospholipid Signaling in Cancer, Neurodegeneration and Cardiovascular Disease, Steamboat Springs, CO. July 31-August 5, 2016. Unfortunately, due to a death in my family, I had to cancel at the last minute.

3.5 The Plan for the Next Reporting Period

Continue as was proposed in the original application. As mentioned above, we have already made substantial progress in Aims 1 and 2 and have also continued to accomplish Aim 3. As discussed above, we have also found an important link between SphK1/S1P and tamoxifen resistance which we intend to pursue further.

4. IMPACT

4.1. The impact on the development of the principal discipline of the project

Hormonal therapies, including selective estrogen receptor modulators and aromatase inhibitors, are the standards of care for treatment of ER positive breast cancer. However, development of resistance to hormone therapies in advanced breast cancer is a major obstacle. Moreover, treatment of TNBC, which has poor prognosis, remains challenging because the tumors are more aggressive and resistant to hormonal therapy (12). Obesity, which has drastically increased in the last decades, has been associated with increased risk for lymph node metastasis, endocrine therapy resistance, larger tumors, death, and for presenting with TNBC (4, 5, 13-16). Several HDAC inhibitors have been developed that restored the efficacy of hormonal therapy in preclinical models and a few have advanced to clinical trials (17, 18), and to a phase III clinical trial that is currently underway (ClinicalTrials.gov identifier: NCT02115282).

In this study we have shown that the pro-drug FTY720 (fingolimod, Gilenya) approved for human use is phosphorylated to the active form, FTY720-P, a histone deacetylase inhibitor that reactivates ERα expression and enhances hormonal therapy for breast cancer. Moreover, oral administration of clinically relevant doses of FTY720 suppressed development, progression, and metastasis of spontaneous breast tumors in transgenic mice on HFD and reversed HFD-induced loss of estrogen and progesterone receptors in advanced carcinoma. We also identified a critical role of the SphK1/S1P axis in tamoxifen resistance. Our work suggests that a multi-pronged attack with FTY720 is a novel combination approach for effective treatment of conventional hormonal therapy-resistant breast cancer and triple-negative breast cancer.

FTY720 has several advantages over available HDAC inhibitors as potential treatments for breast cancer patients: it is an orally bio-available pro-drug; it has already been approved for human use; it regulates expression of only a limited number of genes (a majority related to cholesterol and sphingolipid metabolism) compared to other HDAC inhibitors; it has good pharmacokinetics and a long half life; it suppresses several survival and proliferative pathways; and it is much less toxic, accumulates in tumor tissues, and both the phosphorylated and unphosphorylated forms target important pathways in breast cancer. Hence, we hope that our studies will pave the way for exploration of new clinical trials using FTY720 as a prototype of new adjuvant treatment strategies for hormonal resistant breast cancer. This might be particularly relevant in view of the increase in obesity that is now endemic and in de novo and acquired resistance to hormonal therapy.

4.2. The Impact on Other Disciplines

Although this work may not have a direct impact on other disciplines it might contribute to them, particularly in the treatment of cognitive impairment. HDAC inhibitors have shown promise as a treatment to combat the cognitive decline associated with aging and neurodegenerative disease, as well as to ameliorate the symptoms of depression and posttraumatic stress disorder, among others. Due to its unique features described above and its high brain penetration, FTY720 might be more effective than other HDAC inhibitors as an adjuvant therapy for erasing aversive memories (19). This might also be relevant to suppression of cognitive impairment and neuropathic pain associated with chemotherapy.

4.3. The Impact on Technology Transfer
Nothing to report
4.4. The impact on Society Beyond Science and Technology
Nothing to report

5. CHANGES/PROBLEMS
There are no significant changes in the project or its direction. There is a 3 month delay in the recruitment of a new postdoctoral fellow as my brother had pancreatic cancer and I was away for a significant period in support of my family.

6. PRODUCTS

Publications
1. Newton, J., Lima, S., Maceyka, M., and **Spiegel, S**. Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy. Exp. Cell Res. 333: 195-200, 2015.
2. Maczis, M., Milstien, S., and **Spiegel, S**. Sphingosine-1-phosphate and estrogen signaling in breast cancer. Adv. Biol. Regul. doi: 10.1016, 2015.
3. Nagahashi M, Yamada A, Miyazaki H, Allegood JC, Tsuchida J, Aoyagi T, Huang WC, Terracina KP, Adams BJ, Rashid OM, Milstien S, Wakai T, **Spiegel S**, Takabe K. Interstitial Fluid Sphingosine-1-Phosphate in Murine Mammary Gland and Cancer and Human Breast Tissue and Cancer Determined by Novel Methods. J. Mammary Gland Biol. Neoplasia. 21: 9-17, 2016.
4. Katsuta E, DeMasi SC, Terracina KP, **Spiegel S**, Phan GQ, Bear HD, Takabe K. Modified breast cancer model for preclinical immunotherapy studies. J. Surg. Res. 204: 467-474, 2016.

Abstracts
1. Hait NC, Avni D, Yamada A, Milstien S, Takabe K, **Spiegel S**. FTY720-P is a potent inhibitor of class I histone deacetylases that enhances histone acetylation, reactivates ERα expression, and increases hormonal therapeutic sensitivity of breast cancer. Cancer Research, Abstract 112, DOI: 10.1158/1538-7445. AM2015-112, Published 1 August 2015
2. Maczis AM, Hait NC, Milstien S, **Spiegel S**. Role of Sphingosine-1-Phosphate in Non-Genomic Effects of ERα36 in Breast Cancer. SERLC, Cashiers, NC, November 13-15, 2015.
3. Maczis AM, Hait NC, Milstien S, **Spiegel S**. Role of ERα36 in Sphingosine-1-Phosphate/Sphingosine Kinase 1 Axis in Breast Cancer. Graduate Symposium, Richmond, VA, April 19, 2016.
4. Maczis AM, Hait NC, Milstien S, **Spiegel S**. Role of ERα36 in Sphingosine-1-Phosphate/Sphingosine Kinase 1 Axis in Breast Cancer. 12th Annual Women's Health Research Day, Richmond, VA, April 27, 2016. (Award: Basic Science Research Award)
5. Maczis AM, Hait NC, Milstien S, **Spiegel S**. Sphingosine-1-Phosphate/Sphingosine Kinase 1 Axis Activated by 17B-Estradiol Through Estrogen Receptor alpha splice variant, ERα36, in Breast Cancer. CRR, Richmond, VA, June 17, 2016. (Award: 3rd Place Poster Presentation).

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7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals that have worked on the project

Name: Sarah Spiegel
Project Role: PI – No change

Name: Sheldon Milstien
Project Role: Co-Investigator – No change

Name: Kazuaki Takabe
Project Role: Co-Investigator – Left VCU June 2016, now at Roswell Park, Clinical Chief of Breast Surgery and Breast Disease Site Leader. We will continue our long-standing collaboration but his salary is covered by his new institution.

Name: Melissa Maczis
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID):0000-0002-8610-2475
Nearest person month worked: 12
Contribution to Project: Ms. Maczis joined the lab as a graduate student two years ago. She is well trained to carry out experiments with breast cancer models and also has made a discovery of the function of ERα36 important for acquired resistance to tamoxifen.
Funding Support: Melissa was supported for the first two years as are all graduate students by the School of Medicine. As Ms. Maczis support from the VCU School of Medicine Graduate Program has ended, and she has made such outstanding contributions to all studies, she has been included in this grant as of July 2016.

Name: Nita Hait
Project Role: Collaborator
Researcher Identifier (e.g. ORCID ID): 0000-0002-9433-5498
Nearest person month worked: 5
Contribution to Project: Dr. Hait discovered that FTY720-P is an HDAC inhibitor and has performed the work with the transgenic mice. His involvement in the project has been completed and we already published his work. In May 2016, he left VCU and I am very pleased that he as one of my trainees has obtained an independent position.

Name: Andreia Leopoldino
Project Role: Visiting Scientist
Researcher Identifier (e.g. ORCID ID):0000-0002-8313-4754
Nearest person month worked: 2

Contribution to Project: Dr. Leopoldino was a visiting scientist from Brazil. She came to my lab to gain knowledge in sphingolipid metabolites signaling in inflammation and cancer and is especially interested in the use of FTY720 for cancer treatment.
Funding Support: Dr. Leopoldino had a fellowship from Brazil and returned there Feb 2016.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Yes. Previous active grants have been closed and one new grant is now active.

Previous active grants that have been closed

R01 AI095494-01A (PI:Oskeritzian) 07/27/2012-6/30/2016 0.6 calendar
NIH/NIAID $22,014 (Annual Direct costs)
Project Title: Mast Cell S1P Receptor2 in the Initation and Progression of Chronic Inflammation.
Role: Key Personnel – sub-award University of South Carolina

R01 CA160688-01A (PI: Takabe) 07/09/2012-4/30/2017 0.6 calendar
NIH/DHHS $19,893 (Annual Direct costs)
Project Title: Sphingosine-1-phosphate in breast cancer progression and lymphangiogenesis
Role: Key Personnel – sub-award Surgery/VCU

New Grant

R56 AI125433 (PI:Spiegel) 08/01/2016-07/30/2017 1.2 calendar
NIH/NIAID $250,000 (Annual Direct costs)
Project Title: ORMDL3-ceramide axis in allergic asthma
Role: PI

However, it should be emphasized that these changes do not at all impact the effort on this project that is the subject of this report (W81XWH-14-1-0086).

What other organizations were involved as partners?
Nothing to Report

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9. **APPENDICES**

1. Newton, J., Lima, S., Maceyka, M., **Spiegel, S**. Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy. *Exp Cell Res* 333: 195-200, 2015
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EXPERIMENTAL CELL RESEARCH 333 (2015) 195–200

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journal homepage: www.elsevier.com/locate/yexcr

Review Article

Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy

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ARTICLE INFORMATION

Article Chronology:
Received 26 February 2015
Accepted 28 February 2015
Available online 11 March 2015

Keywords:
Sphingolipid rheostat
Sphingosine-1-phosphate
Sphingosine kinase
Ceramide

Contents

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http://dx.doi.org/10.1016/j.yexcr.2015.02.025

Introduction

Nearly two decades have passed since it was first proposed that regulation of the interconvertible sphingolipid metabolites, ceramide and sphingosine-1-phosphate (S1P), and their opposing signaling pathways are major determinants of cell fate, a concept referred to as the "sphingolipid rheostat". Since then, many reports have substantiated the role of the sphingolipid rheostat in cell fate determination and in the initiation, progression, and drug sensitivity of cancer. Thus, modulation of the rheostat has emerged as a focus for treatment strategies to battle cancer. S1P regulates numerous processes important for cancer including proliferation, transformation, angiogenesis, metastasis, survival, and drug resistance. Ceramide on the other hand has been linked to cell growth arrest and cell death. With the increased understanding of sphingolipid metabolism and signaling, as well as the present focus on therapies designed to modulate the levels of sphingolipids in cancer, it is an appropriate time to re-examine the sphingolipid rheostat concept and determine how it fits within the current knowledge of sphingolipid signaling in cancer.

Sphingolipid metabolism

Sphingolipids are essential constituents of all eukaryotic membranes. They contain a sphingoid base, a fatty amino alcohol of typically 18 carbons, in mammalian cells called sphingosine. *De novo* synthesis of the sphingoid base begins with the condensation of palmitate and serine catalyzed by serine palmitoyl transferase, leading to the formation of dihydrosphingosine (sphinganine), which is then amino-acylated with a chain of 14–32 carbons to form various dihydroceramide species by a family of six (dihydro) ceramide synthases. Dihydroceramides are desaturated to form ceramides and complex sphingolipids, such as glycosphingolipids and sphingomyelin that are built by linking different head groups to the primary hydroxyl group of ceramides. During catabolism, both basal and signal-mediated, these head groups are hydrolyzed, re-generating ceramide. Ceramide is a bioactive lipid in its own right, and can be deacylated by ceramidases to yield sphingosine. Sphingosine, which is not an intermediate in the *de novo* biosynthetic pathway, is also a bioactive molecule and can be phosphorylated by sphingosine kinase (SphK) type 1 and 2 to sphingosine-1-phosphate (S1P), again a potent signaling molecule. S1P can be irreversibly degraded by S1P lyase (SPL) or dephosphorylated to sphingosine, which can then be re-acylated back to ceramide. It is the rapid, compartment-specific interconversion of these three metabolites with distinct effects on cell fate that forms the biochemical basis of the so-called "sphingolipid rheostat".

The sphingolipid rheostat

In 1996, the term "sphingolipid rheostat" was proposed [1] to tie together several seminal findings demonstrating the capacity of S1P and ceramide to differentially regulate cell growth and survival by modulation of opposing signaling pathways [1–3]. This was based on the discoveries that elevation of ceramide induces cell growth arrest and apoptosis [3], whereas S1P production is required for optimal cell proliferation induced by growth factors [4] and suppresses ceramide-mediated apoptosis [1]. Insight that the "sphingolipid rheostat" coordinately regulates the levels of these sphingolipid metabolites to control cell fate emerged from inhibition of SphK leading to decreased S1P and elevated ceramide, and subsequent cell death (Fig. 1). Thus, the sphingolipid rheostat appeared to be a sensing mechanism for cells to regulate their fate in part through the interconversion between S1P and ceramide.

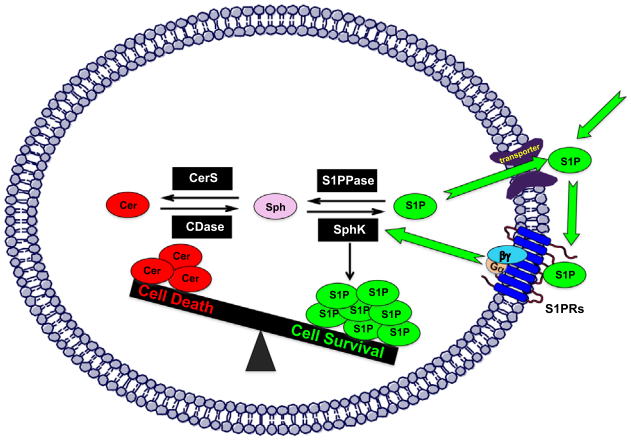

Fig. 1 – The updated sphingolipid rheostat. This schematic cartoon shows important enzymes that regulate the levels of S1P and ceramide and includes "inside-out" signaling by the S1P/S1PR1 axis that can influence actions of the sphingolipid rheostat. CerS, ceramide synthase; CDase, ceramidase; S1PPase, S1P phosphatase; S1PRs, S1P receptors.

In the years since, efforts have been made to elucidate the molecular mechanisms and signaling pathways by which these metabolites exert their effects, and to manipulate the S1P/ceramide balance to direct cells down particular paths for the development of therapeutics targeting the sphingolipid rheostat. In the process, these studies have revealed roles that S1P and ceramide play in the etiology of several debilitating human diseases, particularly cancer, and have clarified the enormous complexity of the interplay between S1P, ceramide, and sphingolipid metabolism, and how this affects complex cellular responses and biological programs. In light of these recent findings, we will revisit whether the S1P/ceramide rheostat concept adequately addresses the complex nature by which sphingolipids affect physiological processes and modulate cell fate, and, accordingly, their role in cancer.

Role of sphingolipid metabolites in cell fate and cancer

Ceramide

Ceramide is a tumor suppressor, promoting intrinsic and extrinsic apoptotic pathways, autophagic cell death, and the inhibition of cell growth, and thus it is not surprising that enzymes responsible for production of ceramide are often altered in cancer resulting in reduction of ceramide accumulation [5]. Moreover, many chemotherapeutic drugs elevate ceramide, and blocking the increase in ceramide provides drug resistance. While specific molecular species of ceramide have been implicated in some of these

pathways, it is unclear whether the specific species itself is required or is merely a reflection of its compartmental- or enzyme-specific generation. For example, it was shown that in glioblastoma tumors, a metabolic shift favoring S1P at the expense of C18 ceramide may be a major contributor to angiogenesis [6]. Although there are numerous pathways affected by ceramide, only a few direct targets have been convincingly identified. The key players in ceramide regulated signaling in cancer are activation of serine/threonine protein phosphatases, such as PP1, PP2A and PP2C, protein kinase Cζ (PKCζ) and inhibition of AKT (reviewed in Ref. [5]). Formation of ceramide-enriched membrane microdomains is a general mechanism by which ceramide can regulate numerous signaling pathways at the plasma membrane or in the outer mitochondrial membrane important for BAX insertion, oligomerization, pore formation and apoptosis.

S1P and its receptors

Within two years of the development of the rheostat concept, the first cell surface G-protein coupled receptor for S1P was discovered [7], followed by the identification of the other members of the S1P receptor family, designated S1PR1–5 [8]. Moreover, intracellular S1P generated by activation of SphK can readily be secreted to act in an autocrine or paracrine manner [9], a paradigm that has been coined inside-out signaling by S1P (Fig. 1) [10]. Signaling through S1PR1–5 fits nicely into the rheostat hypothesis as activation of S1PRs has been shown to promote growth, survival, motility angiogenesis, lymphangiogenesis, and metastasis, important for the pro-cancer activities of S1P [11]. For example, several S1PRs activate the pro-survival ERK and Akt signaling pathways, and S1PR3 activation initiates a signaling cascade through the mTOR pathway that counteracts ceramide-mediated autophagy [12]. Moreover, recent studies have shown that the S1P/S1PR1 axis is at the nexus between NF-κB and STAT3 and connects chronic inflammation to colitis-associated cancer [13]. S1P produced by SphK1 is essential for production of the NF-κB-regulated pro-inflammatory cytokines TNF-α and IL-6, leading to activation of the transcription factor STAT3, and consequent upregulation of its target gene S1PR1 [14]. Reciprocally, S1PR1 maintains STAT3 activation in a malicious feed-forward amplification loop important for colon cancer, lymphoma and glioblastoma [14,15].

S1P transporters

How does S1P, which is made by intracellular SphKs exit cells to activate S1PRs? There is now ample evidence that cells export intercellular S1P into the extracellular environment both through ABC transporters as well as the major facilitator superfamily member, Spinster 2 (Spns2) [10]. The S1P secreted from tumor cells through these transporters can act in an autocrine fashion to promote the growth and motility of the tumors themselves, but more importantly on the tumor microenvironment to enhance angiogenesis and lymphangiogenesis [16], as well as affecting tumor-associated macrophages [14], and may differentially recruit immune cells such as Tregs to block anti-tumor immunity [17].

S1P intracellular targets

Though the majority of known S1P functions are attributable to its action through cell surface S1PRs, recently several intracellular targets of relevance to cancer have been found. The first of these is TRAF2, an essential component in the TNF- α/NF-κB signaling pathway. TRAF2 is an E3 ubiquitin ligase, and it was found that S1P bound to and stimulated its ubiquitin ligase activity [18]. In addition, SphK1 was shown to be required for the TNF-α-induced ubiquitination of RIP1 and subsequent activation of NF-κB, a pro-growth mediator. Interestingly, another group showed that SphKs were dispensable in bone marrow-derived macrophages for TNF-α-induced activation of NF-κB, though even in wild type macrophages TNF-α did not increase S1P levels, suggesting alternative mechanisms for the stimulation of TRAF2 activity [19]. S1P, produced by SphK2, was shown to bind to and inhibit histone deacetylases 1 and 2, leading to increases in histone acetylation [20]. Further support for this notion that HDACs are intracellular targets of S1P emerged from a recent study in *Drosophila*, which have no identified S1PRs, showing that increased nuclear S1P caused decreased HDAC activity and increased histone acetylation, and importantly suppressed dystrophic muscle degeneration [21]. Furthermore, the pro-drug FTY720 is also phosphorylated in the nucleus by SphK2 and FTY720-phosphate is a potent class I HDAC inhibitor [22], which might explain its potent anti-cancer effects.

Targeting S1P metabolic enzymes to modulate the sphingolipid rheostat and cancer

Recently several excellent reviews discussed how targeting specific ceramide metabolic enzymes regulates the sphingolipid rheostat to amplify tumor suppressive activities of ceramide and consequently cell fate, and highlights the usefulness of ceramide-based therapeutics for treatment of cancer [5,23]. Therefore, we will mainly focus in this section on effects of targeting S1P metabolism.

SphK1

In many cancers, elevated levels of SphK1 are an independent predictor of mortality, and strongly correlate with poor prognosis, reduced overall survival, and advanced tumor stages [11,24]. However, no mutations in SphKs have been identified, suggesting that it is the regulation of SphK activity, and hence a potential for S1P "cellular addiction", that is responsible for SphK1's "oncogenic" role [25]. There is some evidence to support this notion: 1) in cell culture, expression of SphK1 and S1P levels dictate resistance to cytotoxic drugs and radiation; 2) in animal models, overexpression of SphK1 and formation of S1P leads to aggressive tumors, and inhibition of SphK1 reverses drug resistance and enhances sensitivity to radiotherapy; 3) SphK1 is overexpressed in many types of cancer and high levels of SphK1 correlate with poor outcomes in patients. Because of these observations, multiple drugs targeting SphK1 have been designed. The "first generation" inhibitors such as N,N-dimethyl-sphingosine and SK1-II, with poor potency and selectivity between SphK isoforms, as well as SK1-I which specifically targets SphK1, though with low potency, showed promise in preclinical animal models. However

studies with the "second generation" of SphK1 inhibitors, such as PF-543, which are much more potent and are highly selective, have limited to no success in inducing apoptosis [26,27], even though in all cases S1P levels were reduced and in two of these studies ceramide levels were concomitantly elevated [6,28]. Why then, if the rheostat postulates that a reduction in S1P and rise in ceramide should increase cell death, are these SphK1 inhibitors ineffective? There are several potential explanations for this: first, reduction of SphK1 levels due to proteasomal degradation may be critical [29]. Second, inhibitors might affect SphK1 activity in different subcellular locations and only those that affect cellular S1P *vs*. sphingosine and ceramide more profoundly are effective compared to those that simply inhibit SphK1. Second generation inhibitors, with their much higher specificity and potency, do not have strong effects on changing the sphingolipid rheostat and only increase ceramide levels at an order of magnitude greater concentration than their K_i values [28]. This further substantiates the notion that the sphingolipid rheostat is a critical component, not only reduction in the levels of S1P. Hence, in light of this, it might be appropriate to consider a more broad-spectrum approach to therapeutics to induce the apoptotic benefits that dictate chemotherapeutic efficiency. Third, as indicated by Abu-husain et al. [6], S1P produced by cancer cells may mainly act in a paracrine manner in the tumor microenvironment that is important for angiogenesis and lymphangiogenesis but does not affect tumor growth itself. It is important to note that all of the studies reported so far with second generation SphK1 inhibitors utilized cultured cancer cells, and therefore it will be important to examine their effects in animal cancer models. As more and more SphK1 drugs are developed with fewer off target effects, establishing how perturbations in S1P/sphingosine balance affects ceramide should become easier. These ventures should also be significantly aided with a variety of SphK1 structures that have been published [30], and with the recent development of a high-throughput assay to screen SphK inhibitors [31].

SphK2

In contrast to SphK1, the actions of SphK2 remain poorly characterized and much less is known about its biology and roles in cancer and other diseases. However, SphK2 is critical to the function of one of the few FDA approved sphingosine analog drugs, FTY-720, as it phosphorylates it to the "active" form that acts on S1PRs (except S1PR2) [32] and inhibits histone deacetylases [22]. The difficulty in targeting SphK2 in rheostat modulation therapies is that its roles in regulating sphingolipid metabolism are not well understood. SphK2 is present in several subcellular compartments, and there are conflicting data regarding its role in cancer development. Moreover, inhibitors targeting its activity have not been as successfully developed as those for SphK1. Compound ABC294640 (SphK2 $K_i = 9.8\ \mu M$) has shown promise in reducing cancer cell growth *in vitro* and in mouse models of cancer [33,34]. However, this compound has also been linked to potential off target anti-estrogenic effects [35]. In another example, SLR080811 (SphK2 $K_i = 1.3\ \mu M$) showed a reduction of the levels of S1P in cells, though it had no anti-proliferative properties, and when administered to mice, raised blood S1P levels [36], confounding evaluation of its effectiveness as a chemotherapeutic in mice cancer models. Clearly, targeting SphK2 in the treatment of disease is still in its infancy and will require significant efforts to substantially increase the potency and specificity of inhibitors. Unfortunately, unlike SphK1, little is also known of the structure of SphK2 and pharmacophore design for SphK2 is complicated by the necessity to cross secondary subcellular membrane barriers to reach the target.

S1P lyase and S1P phosphatases

As the terminal step in irreversible catabolism of all sphingolipids, SPL controls levels of S1P and other bioactive sphingolipid metabolites, and is also the link between sphingolipid and phospholipid metabolism. Indeed, deletion of SPL not only increases S1P levels but also sphingosine and ceramide, probably due to reutilization of the sphingosine backbone for ceramide synthesis. Not surprisingly, knockout mice have severely altered lipid homeostasis, aberrant S1P signaling, and inflammatory responses leading to early death [37,38]. SPL levels are downregulated in various human cancers and inversely correlated to clinical outcomes and resistance to treatment, further supporting a role for S1P in cancer development. Several novel findings leading to mechanistic actions of SPL in tumorigenesis and chemoresistance have recently been described. Deletion of intestinal SPL promoted colon carcinogenesis through the S1P/S1PR1 axis and activation of STAT3. STAT3 in turn enhanced expression of specific miRs that target the anti-oncogenes PTEN (a lipid phosphatase that negatively regulates the PI3K/AKT pathway) and cylindromatosis (CYLD; a deubiquitinating enzyme that negatively regulates NF-κB) [39]. Interestingly, upregulation of SPL levels by consumption of sphingadienes, plant-type sphingolipids that cannot be converted to S1P, was able to enhance the metabolism of S1P attenuating STAT3 signaling, cytokine production, and tumorigenesis [39]. SPL deficiency or its inhibition has also been associated with elevated nuclear S1P levels and reduced HDAC activity that in turn induced dysregulation of Ca^{2+} homeostasis [40], and upregulation of several S1P transporters, including multi-drug resistant proteins, contributing to chemoresistance [41].

In addition to SPL, S1P levels are also reversibly regulated by two S1P phosphatases (SGPP1 and SGPP2) that dephosphorylate S1P to sphingosine that can then be used for ceramide formation. This places S1P levels under dual control, with one pathway removing sphingolipids from the signaling pool and the other shifting the rheostat balance from the proliferative effects of S1P to the pro-apoptotic effects of sphingosine and ceramide accumulation. Similar to SPL, there is evidence that S1P phosphatase expression is downregulated in several types of cancer. Closer examinations of these studies reveals that increased S1P content in the tumors was correlated with decreased SGPP2 expression and increased SphK1, supporting the notion of coordinated regulation of sphingolipid metabolism [6].

Modified rheostat paradigm: addition of the S1P/S1PR axis

The field of cancer research has embraced the concept of the sphingolipid rheostat. As more and more studies involving ceramide and S1P signaling attempt to use the rheostat model to explain their findings, and as the signaling mechanisms by which these sphingolipid metabolites exert their control on cell fate becomes more complex, and additional proteins that regulate sphingolipid metabolism are discovered, the need for a more

nuanced model has become apparent. For example, although initially elevation of ceramide was linked to cell growth inhibition and reduction of tumor growth, more recent studies suggest that ceramides with different fatty acid chain lengths might play distinct functions. *De novo*-generated C18- and C16-ceramides by CerS1 and CerS6 play opposing proapoptotic and prosurvival roles in the regulation of tumor growth, respectively [42]. Whether a specific acyl chain species is required for apoptosis, for example by N-acyl chain-specific binding to effector proteins, or whether the N-acyl chain species generated is merely a reflection of the enzyme- and/or compartment-specific generation of ceramide, has yet to be elucidated.

The molecular roles of S1P in the rheostat have become more complex. It is generally accepted that S1P is a pro-survival factor and many studies have demonstrated that S1P acts through cell surface S1PRs through "inside-out" signaling to promote cancer growth, progression and metastasis [43]. S1P does this through the autocrine promotion of tumor growth and importantly, in a paracrine manner by enhancing angiogenesis, lymphangiogenesis, recruitment of pro-tumor immune cells, and affecting the tumor microenvironment. This suggests that the "sphingolipid rheostat" concept should be modified to include this "inside-out" signaling (Fig. 1). Furthermore, a recent study showed that S1P can also act in a feed-forward, "outside-in" signaling in a paracrine fashion. Namely, S1P acting through S1PRs has been shown to stimulate SphK1 transcription *via* the transcription factor AP-1, which is composed of c-Fos and c-Jun [44]. This positive feedback loop maintains sustained activation of the SphK1-S1P axis and increased fibronectin expression leading to initiation and progression of diabetic nephropathy [44], and could also contribute to the pathogenesis of other diseases including cancer. Moreover, activation of Gq induced plasma membrane translocation of SphK1 and cross-activation of S1PRs [45]. Hence, increased SphK1 activity and increases in S1PR synthesis or their activations, completes this positive feedback amplification loop.

These more recent studies suggest that the enzymes of the rheostat do not just function by directly changing the fate of the sphingoid base (*i.e.* ceramide *vs.* S1P) as initially conceived in the rheostat model, but also by the roles these metabolites have in myriad, often opposing, signaling pathways. While the initial rheostat model was based on the relative intracellular levels of ceramide and S1P determining signaling and cell fate, we now know that the localized production, secretion, and signaling of these metabolites have a profound effect on tumor outcomes. Our new, more nuanced understanding of the sphingolipid rheostat must be taken into consideration as we design, test, and implement new chemotherapeutics targeting this axis for cancer treatment. More work is needed to understand alterations that occur in the complex sphingolipid pathways during cancer development and progression, and their relationship to the Warburg effect and the metabolic shift from oxidative phosphorylation to the synthesis of lipids and biomass essential for increased cellular proliferation [46].

Acknowledgements

This work was supported by U.S. NIH Grants R01 GM043880 and R01 CA61774 (S.S.), 5T32 HL094290 (J.N.), and 1K22 CA187314 (S. L.) and U.S. Department of Defense W81XWH-14-1-0086 (S.S.)

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Advances in Biological Regulation 60 (2016) 160–165

Contents lists available at ScienceDirect

Advances in Biological Regulation

journal homepage: www.elsevier.com/locate/jbior

Sphingosine-1-phosphate and estrogen signaling in breast cancer

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ARTICLE INFO

Article history:
Received 23 September 2015
Received in revised form 28 September 2015
Accepted 28 September 2015
Available online 19 October 2015

Keywords:
Sphingosine-1-phosphate
Sphingosine kinase
Estradiol
Breast cancer
FTY720/Fingolimod

ABSTRACT

Breast cancer remains the most common malignant disease in women. The estrogen receptor-α (ERα) and its ligand 17β-estradiol (E_2) play important roles in breast cancer. E_2 elicits cellular effects by binding to ERα in the cytosol followed by receptor dimerization and translocation to the nucleus where it regulates gene expression by binding to ERE response elements. However, it has become apparent that E_2 also exerts rapid non-genomic effects through membrane-associated receptors. There is emerging evidence that this induces formation of the bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P). S1P in turn has been implicated in many processes important in breast cancer progression. One of the enzymes that produce S1P, sphingosine kinase 1 (SphK1), is upregulated in breast cancer and its expression has been correlated with poor prognosis. This review is focused on the role of the SphK/S1P axis in estrogen signaling and breast cancer progression and will discuss new therapeutic approaches targeting this axis for breast cancer treatment.

Contents

Abbreviations: ERα, estrogen receptor α; EGFR, epidermal growth factor receptor; ERE, estrogen response element; ERK, extracellular signal regulated kinase; E_2, 17β-estradiol; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor 2; MAPK, mitogen activated protein kinase; PHB2, prohibitin 2; SphK, sphingosine kinase; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; TNBC, triple negative breast cancer; TRAF2, TNF receptor-associate factor 2.

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http://dx.doi.org/10.1016/j.jbior.2015.09.006
2212-4926/

1. Introduction

The estrogen receptor-α (ERα) and its ligand 17β-estradiol (E_2) play important roles in breast cancer. Most of the canonical genomic effects of binding of E_2 to ERα are mediated by nuclear transcriptional regulation. However, E_2 also exerts rapid non-genomic signaling through membrane-associated receptors many of them resulting from increased formation of the bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P). S1P and sphingosine kinases (SphKs) that produce it have been implicated in many processes important in breast cancer progression. In this review, we discuss the role of the SphK/S1P axis in estrogen signaling and breast cancer progression and also some new therapeutic approaches to potentially target this axis for breast cancer treatment.

1.1. Formation and metabolism of S1P

It has long been known that sphingolipid metabolism generates metabolites with important functions. The best characterized are ceramide, the backbone of all sphingolipids, its breakdown product sphingosine, and S1P. S1P metabolism has been discussed in many reviews (Hannun and Obeid, 2008; Maceyka and Spiegel, 2014; Shamseddine et al., 2015) and is only briefly outlined here. Two sphingosine kinases, known as SphK1 and SphK2, catalyze the phosphorylation of sphingosine to S1P, which is irreversibly cleaved by S1P lyase to phosphoethanolamine and a fatty aldehyde or dephosphorylated back to sphingosine by several phosphatases which then can be reutilized for ceramide and sphingolipid formation. Tissue levels of S1P are thus determined by the balance between activity of SphKs and S1P lyase and phosphatases.

1.2. S1P signaling

1.2.1. S1P and its receptors

S1P has important roles in regulation of a wide variety of complex biological processes important for breast cancer progression (Carroll et al., 2015; Maceyka and Spiegel, 2014). Most of these actions are mediated by binding to a family of five specific cell surface receptors (S1PR1-5) (Maceyka and Spiegel, 2014). Numerous stimuli, including hormones such as estradiol (E_2), rapidly activate SphK1 and/or SphK2 to transiently increase intracellular S1P levels in specific pools. S1P produced mainly by activated SphK1 can then be secreted by Spns2, a member of the major facilitator superfamily of non-ATP-dependent transporters or by ABC transporters ABCA1, ABCC1, and ABCG2 (Nishi et al., 2014; Takabe and Spiegel, 2014). S1P in turn activates its receptors in an autocrine or paracrine manner known as 'inside-out' signaling of S1P (Hobson et al., 2001; Takabe et al., 2008). Physiological responses regulated by S1P depend on the spectrum of ubiquitously but differentially expressed S1PRs and the variety of G proteins they are coupled to. Thus, many signaling pathways downstream of S1PRs that have been linked to cancer progression have been shown to be activated depending on the cell type, including MAPKs, phospholipase C, adenylate cyclase, and Rac/PI3K/Akt, to name a few (Pyne et al., 2014; Takabe et al., 2008). Moreover, various types of cancer cells differentially express different sets of S1PRs, thus providing S1P with the ability to regulate numerous cellular processes important for breast cancer, including growth, survival, migration, invasion, inflammation, angiogenesis, and lymphangiogenesis (Nagahashi et al., 2014).

1.2.2. Intracellular actions of S1P

While it has long been suspected that S1P also has intracellular actions that are independent of S1PRs, only recently have several intracellular targets been identified that are likely to be important in the context of cancer. We found that S1P, but not dihydro-S1P, produced by SphK1 activated by TNF directly binds to and activates the E3 ubiquitin ligase activity of TNF receptor-associate factor 2 (TRAF2), an important component in NF-κB signaling (Alvarez et al., 2010). NF-κB regulates transcription of pro-survival or anti-apoptosis genes, thus identifying one of the mechanisms for the pro-survival actions of S1P in cancer progression. Interestingly, in contrast to SphK1, which is localized to the cytosol, SphK2 is mainly in the nucleus of most types of cells. We showed that nuclear S1P produced by ERK/MAPK-dependent activation of SphK2 is an endogenous inhibitor of histone deacetylases (HDACs) (Hait et al., 2009). Since SphK2 is present in repressor complexes together with HDACs in the nucleus of breast cancer cells (Hait et al., 2009), the S1P it produces inhibits HDAC activity resulting in enhanced transcription of specific target genes. This was the first indication that nuclear sphingolipid metabolism is involved in epigenetic regulation. Another link between nuclear S1P and gene expression was recently reported by Ogretmen and colleagues who discovered that S1P binds to hTERT and stabilizes telomerase at the nuclear periphery by allosterically mimicking hTERT phosphorylation. In murine xenografts, inhibitors of SphK2 decreased tumor growth and overexpression of wild-type hTERT in cancer cells, but not a hTERT mutant that was unable to bind S1P, restored tumor growth (Panneer Selvam et al., 2015). Their results suggest that S1P promotes telomerase stability and telomere maintenance important for cancer cell proliferation and tumor growth. In the mitochondria, SphK2 produces S1P that binds to prohibitin 2 (PHB2), a protein that regulates mitochondria assembly and function. Deleting SphK2 or PHB2 induced a mitochondrial respiration defect through cytochrome c oxidase (Strub et al., 2011) and may be important for the well-known Warburg metabolism of cancer cells. In this regard, it was suggested that SphK1, but not SphK2, functions to maintain the Warburg effect and cell survival (Watson et al., 2013).

1.3. Role of estrogen in breast cancer

Breast cancer is the most common cancer among women worldwide and occurs in about 1 in 8 women in the US (http://www.cancer.org, last accessed June 10, 2015). The estrogen receptor-α (ERα) plays an important role in breast cancer pathogenesis and progression (McDonnell and Norris, 2002). Patients with tumors that express ERα are termed "ERα-positive" and those lacking ERα are termed "ERα-negative". The majority of human breast cancers start out as estrogen-dependent because they are derived from cells that express ERα (Saha Roy and Vadlamudi, 2012). The steroid hormone, E_2, interacts directly with estrogen-specific cytoplasmic/nuclear receptors, ERα66 and ERα46 (Marino et al., 2006). ERα66 is the main ERα responsible for ERα-positive breast cancer responses to E_2. ERα46 is a 46 kDa splice variant that also functions in association with ERα66. The canonical pathway by which E_2 elicits cellular effects is initiated by binding to ERα in the cytosol followed by homo- or hetero-dimerization and translocation to the nucleus. In the nucleus, ERα dimers function as transcription factors by binding to specific response elements (ERE) on DNA, and either activating or repressing transcription (Mangelsdorf et al., 1995). These genomic responses are slow and take days to induce effects through translational events. E_2 also initiates rapid non-genomic responses, taking minutes to cause an effect through a membrane-associated 36 kDa splice variant (ERα36) of ERα66 (Marino et al., 2006), or through a G protein-coupled receptor (GPR30) (Filardo et al., 2007). Ample evidence has accumulated suggesting an important role for S1P in E_2-mediated signaling (Sukocheva and Wadham, 2014).

1.4. Role of the SphK1/S1P axis in ER signaling

Our initial demonstration that overexpression of SphK1 in human breast cancer cells promoted tumorigenesis and neovascularization when implanted in nude mice (Nava et al., 2002) was followed by numerous reports confirming the importance of SphK1 and formation of S1P as anti-apoptotic and growth-promoting factors in breast cancer (Carroll et al., 2015; Newton et al., 2015; Pyne et al., 2014; Sukocheva and Wadham, 2014; Truman et al., 2014). Moreover, expression of SphK1 has been shown to correlate with poor prognosis in breast cancer patients (Pyne et al., 2014; Ruckhaberle et al., 2008).

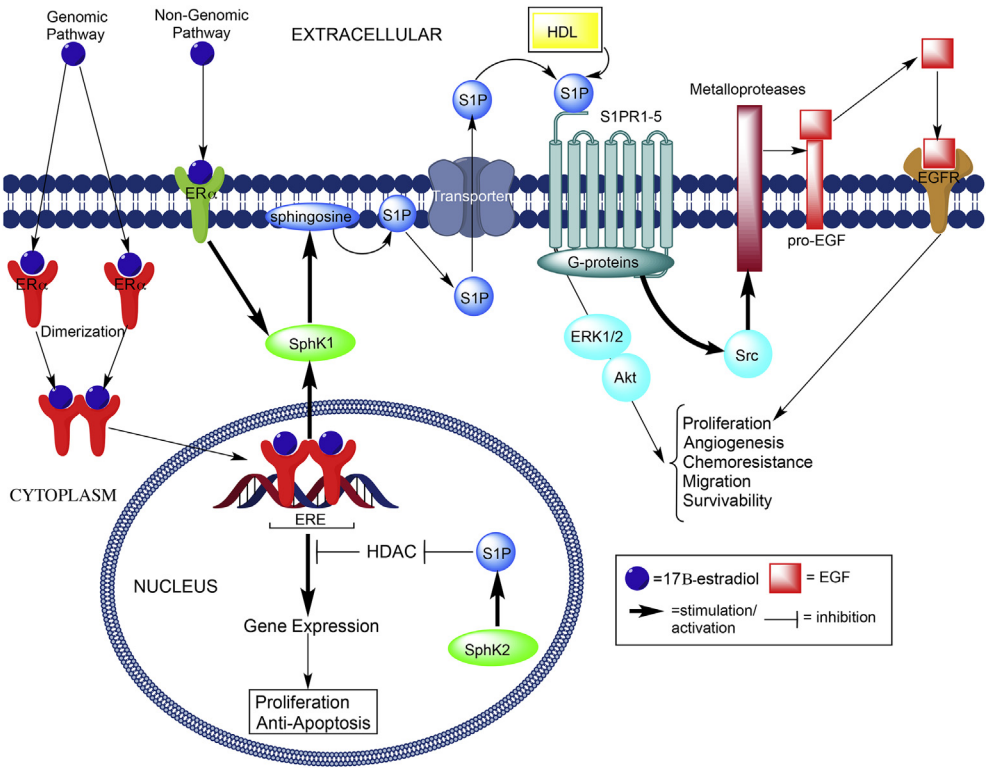

Fig. 1. Role of the SphK/S1P axis in signaling pathways initiated by E_2. Binding of E2 to cytosolic ERα induces its dimerization and translocation to the nucleus where it associates with estrogen response elements (ERE) to regulate gene expression. E_2 can also signal through cell surface E_2 receptors to initiate rapid non-genomic effects that include activation of SphK1 and production of S1P. After export of this S1P by transporters, it activates S1PRs (such as S1PR3) leading to downstream signaling that regulates many processes important for breast cancer progression including processing of pro-EGFR by metalloproteinases. EGF then stimulates EGFR-mediated signaling important for cell growth (Sukocheva et al., 2013). S1P produced in the nucleus by SphK2 is an endogenous inhibitor of HDACs (Hait et al., 2009).

E_2 activates SphK1 in breast cancer cells and increases formation and export of S1P (Sukocheva and Wadham, 2014; Takabe and Spiegel, 2014). Increased SphK1 activity correlates with enhanced cell growth, and also is required for E_2-dependent activation of MAPK and intracellular Ca^{2+} mobilization in ERα-positive MCF-7 breast cancer cells (Sukocheva et al., 2006).

Anti-estrogen therapy with tamoxifen or aromatase inhibitors is the treatment of choice for ERα-positive breast cancer. Unfortunately, loss of ERα expression leading to resistance to hormonal therapies is common and hormonal therapies are not effective in ERα-negative breast cancers. One of the mechanisms by which breast cancer cells become resistant is a switch to growth factor-dependent growth. Intriguingly, activation of ERα by E_2 activates EGFR by a "criss-cross" transactivation process important for E_2-dependent growth that requires ERα, activation of SphK1, production and secretion of S1P that activates S1PR3, leading to enhanced processing of pro-EGFR and activation of EGFR (Sukocheva et al., 2006) (Fig. 1). This also caused increased localization of EGFR in endosomes to delay its degradation and direct it for recycling for continuous proliferative signaling (Sukocheva et al., 2009). Interestingly, SphK1 mRNA is also increased after E_2 treatment, suggesting that SphK1 is transcriptionally regulated by ERα66. Transactivation to EGFR was also detected in T47D ERα-positive cells in response to E_2 treatment, but not in ERα-negative SK-BR-3; whereas, S1P was able to transactivate the EGFR in both ERα-positive and ERα-negative cells (Sukocheva et al., 2013). This suggests that both E_2 and S1P are critical components in the transactivation of EGFR in the transition from E_2-dependent growth to growth-factor-dependent growth. Moreover, SphK1 was required for EGF-induced breast cancer migration, proliferation, and cell survival and both ERα and GPR30 have been implicated in initiation of this signaling (Sukocheva and Wadham, 2014). However, only E_2 and not EGF stimulated export of S1P via ABCC1 and/or ABCG2 from breast cancer cells in an ERα-dependent manner (Takabe et al., 2010). Although Spns2, another bona fide S1P transporter, has been shown to export S1P from cells (Nishi et al., 2014; Takabe and Spiegel, 2014) and to be important in inflammatory and autoimmune diseases in mouse models (Donoviel et al., 2015), its involvement in breast cancer has not yet been investigated.

1.5. S1P in development of tamoxifen resistance

Tamoxifen is an anti-estrogen drug that binds to ERα, preventing estrogen binding, thereby causing cell growth arrest in breast cancer cells that are estrogen-dependent. Previous studies have shown that when patients with ERα-positive breast cancer are treated with tamoxifen for 5 years, the rate of cancer recurrence is reduced by 39 percent and breast cancer mortality is decreased by about one-third throughout the first 15 years (Davies et al., 2011). Unfortunately, half of these patients will ultimately fail therapy due to acquired resistance. Moreover, breast cancer in patients whose tumors do not express ERα, progesterone receptor, and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2), termed triple-negative breast cancer (TNBC), is aggressive with high recurrence, metastatic, and mortality rates (Bayraktar and Gluck, 2013). These patients do not respond to hormonal therapy due to *de novo* (intrinsic) resistance and have limited treatment options.

The SphK1/S1P/S1PRs axis has been implicated in the development of tamoxifen resistance or acquired (extrinsic) chemoresistance. SphK1 expression and activity were shown to be elevated in acquired-tamoxifen resistant ERα-positive MCF7 cells, and SphK1 inhibition or downregulation restored the anti-proliferative and pro-apoptotic effects of tamoxifen (Sukocheva et al., 2009; Watson et al., 2010). In a cohort of 304 ERα-positive breast cancer patients, SphK1 expression correlated with tamoxifen resistance (Pyne et al., 2012). Moreover, high SphK1 and ERK1/2 expression in tumors of ERα-positive breast cancer patients, high S1PR1, but not S1PR2, expression, and higher expression of S1PR1/3 and ERK1/2 were all found to be associated with shorter time to recurrence on tamoxifen (Watson et al., 2010). These correlations suggest that the SphK1/S1P/S1PR1/3 axis and ERK1/2 may cooperate to promote ERα-positive breast cancer progression and resistance to anti-estrogen therapies.

1.6. Inhibition of the ER/S1P axis

Inhibitors that effectively target the ERα/S1P axis could also potentially be useful as new therapies for breast cancer. Numerous studies have shown the inhibitors of SphK1 decrease cancer cell growth and survival and also sensitize them to chemotherapeutics. For example, the non-selective SphK1/2 inhibitor, SKI-II, has been shown to abrogate ERα signaling, likely acting both as a SphK inhibitor and in a similar manner as tamoxifen by directly binding to the ERα and blocking binding of E_2 (Antoon et al., 2011a). Moreover, SphK1 inhibition by siRNA knockdown or treatment with SKI-5C sensitizes TNBC cells to chemotherapeutic drugs (Datta et al., 2014). However, fewer studies have shown the utility of SphK1 inhibitors *in vivo*. We found that treatment mice bearing syngeneic breast tumors with the specific SphK1 inhibitor SK1-I not only suppressed S1P levels in the tumor and circulation, but importantly reduced tumor burden and metastases to lymph nodes and lungs (Nagahashi et al., 2012). Growth of MDA-MB-468 xenograft tumors in mice was significantly inhibited by the SphK1/2 inhibitor SKI-II and the tyrosine kinase inhibitor gefitinib when used in combination, but not as single agents (Martin et al., 2014). Although inhibition of SphK2 has also been reported to reduce tumorigenesis (Antoon et al., 2011b; Liu et al., 2013), further studies are needed to insure that these effects are solely dependent on inhibition of SphK2 activity.

We and others found that the multiple sclerosis pro-drug FTY720/fingolimod, a sphingosine analog that is phosphorylated mainly by SphK2 to a S1P mimetic *in vivo*, has pleiotropic anti-cancer actions in breast cancer cells and in animal models. First, FTY720 has anti-proliferative actions in many types of cancer cells without affecting normal cells (Romero Rosales et al., 2011). Moreover, FTY720 is a substrate and thus a competitive inhibitor of SphK1 and SphK2, decreasing levels of pro-

survival S1P and increasing levels of pro-apoptotic sphingosine (Pyne et al., 2014). Phosphorylated FTY720 (FTY720-P) is also a functional antagonist of and downregulates S1PR1, which interferes with activation of NF-κB and STAT3, and inhibits neovascularization in B cell-derived tumors (Deng et al., 2012; Lee et al., 2010; Liu et al., 2012) and colorectal cancer (Liang et al., 2013; Nagahashi et al., 2014). Furthermore, in breast cancer cells, FTY720-P produced in the nucleus by nuclear SphK2 is a potent inhibitor of class 1 HDACs that enhances histone acetylations and regulates expression of a restricted set of genes important for cancer progression, independently of its known effects on canonical signaling through S1PR1 (Hait et al., 2015). Importantly, in ERα-negative human and murine breast cancer cells and in ERα-negative syngeneic breast tumors, FTY720 activated re-expression of silenced ERα, which restored the ability of the anti-estrogen drug tamoxifen to block breast cancer proliferation and enhance apoptosis (Hait et al., 2015). Because a high fat diet and associated obesity are now endemic and associated with worse prognosis in breast cancer, we also investigated the effect of FTY720 administration on increased tumorigenesis in high fat diet fed mice. FTY720 significantly impaired development, progression and aggressiveness of spontaneous breast tumors in MMTV-PyMT transgenic mice and also reduced HDAC activity and restored expression of estrogen and progesterone receptors induced by the high fat diet (Hait et al., 2015). Taken together, these results provide further support the notion that FTY720 deserves consideration as a new therapeutic for treatment of both hormonal therapy-resistant breast cancer and triple-negative breast cancer.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgments

This work was supported by grants from Department of Defense BCRP Program Award W81XWH-14-1-0086 (S. Spiegel).

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J Mammary Gland Biol Neoplasia (2016) 21:9–17
DOI 10.1007/s10911-016-9354-7

Interstitial Fluid Sphingosine-1-Phosphate in Murine Mammary Gland and Cancer and Human Breast Tissue and Cancer Determined by Novel Methods

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Received: 8 January 2016 / Accepted: 9 May 2016 / Published online: 19 May 2016

Abstract The tumor microenvironment is a determining factor for cancer biology and progression. Sphingosine-1-phosphate (S1P), produced by sphingosine kinases (SphKs), is a bioactive lipid mediator that regulates processes important for cancer progression. Despite its critical roles, the levels of S1P in interstitial fluid (IF), an important component of the tumor microenvironment, have never previously been measured due to a lack of efficient methods for collecting and quantifying IF. The purpose of this study is to clarify the levels of S1P in the IF from murine mammary glands and its tumors utilizing our novel methods. We developed an improved centrifugation method to collect IF. Sphingolipids in IF, blood, and tissue samples were measured by mass spectrometry. In mice with a deletion of SphK1, but not SphK2, levels of S1P in IF from the mammary glands were greatly attenuated. Levels of S1P in IF from mammary tumors were reduced when tumor growth was suppressed by oral administration of FTY720/fingolimod. Importantly, sphingosine, dihydrosphingosine, and S1P levels, but not dihydro-S1P, were significantly higher in human breast tumor tissue IF than in the normal breast tissue IF. To our knowledge, this is the first reported S1P IF measurement in murine normal mammary glands and mammary tumors, as well as in human patients with breast cancer. S1P tumor IF measurement illuminates new aspects of the role of S1P in the tumor microenvironment.

Keywords Cancer · Endothelial cells · Interstitial fluid · Mass spectrometry · Sphingolipids · Sphingosine-1-phosphate

Introduction

The tumor microenvironment is a determining factor in cancer biology and progression [1]. Although it has been long known that the lymphatic system is the initial pathway for metastasis in many cancers including mammary cancer, recent findings

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suggest new mechanisms for how cancer cells gain access to the lymphatic system and how they manipulate their microenvironment to establish metastasis. An increasing number of proteins in the tumor microenvironment are now known to play important roles in tumor progression [2–4]. Interstitial fluid that bathes the tumor and stromal cells is considered as an important part of the tumor microenvironment not only as the initial route of metastasis, but also as a supplier of factors that promote tumor metastasis.

Sphingosine-1-phosphate (S1P) is a potent bioactive signaling molecule that regulates many physiological and pathological processes involved in immune cell trafficking, inflammation, vascular homeostasis, and cancer progression [5–8]. S1P is generated by sphingosine kinases (SphK1 and SphK2), and is then secreted, exerting its functions by binding to five specific G protein–coupled receptors (S1PR1–5) in autocrine, paracrine, and/or endocrine manners, a process known as "inside-out" signaling [9–11]. "Inside-out" signaling refers to the process by which S1P produced inside cells is secreted by transporters and signals through its receptors on the outside of cells. The "inside-out" signaling of S1P plays important roles in cancer cell pathophysiology [12]. Though we have shown that SphK1 is the significant contributor to extracellular S1P while SphK2 contributed to intracellular S1P of mammary cancer cells [12], to date the relative contribution of each SphK to secreted S1P has never been definitively demonstrated in an in vivo setting.

Recently studies from our laboratory have demonstrated that S1P produced by SphK1 in cancer cells promotes mammary cancer progression by stimulating angiogenesis, lymphangiogenesis, and subsequently lymph node metastasis [13], We have also shown that S1P produced by up-regulation of SphK1 and subsequent activation of the S1PR1 receptor play an essential role in maintaining persistent activation of the important transcription factors NF-kB and Stat3 in a feedforward amplification loop that links chronic inflammation and colitis associated carcinogenesis [14]. Despite this emerging understanding of importance of S1P in cancer cell signaling, the role of S1P in the tumor microenvironment, particularly in the interstitial fluid (IF), remains unclear. This is in part because of difficulties presented by collecting and analyzing IF, a barrier that once surmounted, is expected to provide important insights into the tumor microenvironment and how tumors develop and respond to therapy.

Here we introduce simple and reproducible methods to measure the levels of sphingolipids including S1P in small volume of interstitial fluid from healthy mammary glands and tumor using a modified centrifugation method combined with liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Using our new method, we are able for the first time to demonstrate the contributions of SphK1 and SphK2 to secreted S1P in vivo, and have been able to provide definitive evidence that S1P is increased in breast tumor interstitial fluid and that this increase is ameliorated by treatment with the prodrug FTY720, concomitantly with suppression of tumor growth.

Materials and Methods

Reagents

Internal standards were purchased from Avanti Polar Lipids (Alabaster, AL) and added to samples in 20 μl ethanol:methanol:water (7:2:1) as a cocktail of 500 pmol each. The HPLC grade solvents were obtained from VWR (West Chester, PA).). FTY720 was from Cayman Chemical Company (Ann Arbor, MI).

Animals

All animal studies were conducted in the Animal Research Core Facility at VCU School of Medicine in accordance with institutional guidelines. Experiments without breast tumor implantation utilized SphK1$^{-/-}$ and SphK2$^{-/-}$ mice since they are well characterized, and because we have previously found that SphK2$^{-/-}$ mice demonstrate compensatory higher expression of SphK1 in the tissues [14]. These knockout mice were kindly provided by Dr. Richard L. Proia of National Institute of Diabetes and Digestive and Kidney Diseases [15, 16]. We obtained each knockout mouse with littermate WT from heterozygous parents. Experiments with 4T1 breast tumor implantation, used syngeneic Balb/c female mice at 8–10 weeks of age (Harlan, Indianapolis, IN).

Tumor Growth

4T1-luc2 cells, a mouse mammary gland derived adenocarcinoma cell line that has been engineered to express luciferase (Caliper Life Sciences, Perkin Elmer, Waltham, MA), were cultured in RPMI Medium 1640 with 10 % fetal bovine serum. 4T1-luc2 cells (1×10^5 cells in 10 μl RPMI) were implanted in the 2nd chest mammary gland under direct vision as previously described [13, 17]. The tumor burden of 4T1-luc2 cell tumors was determined by measurement of bioluminescence with the IVIS Imaging System (Xenogen, Perkin Elmer). Where indicated, tumor-bearing mice were randomized 2 days after implantation into two treatment groups treated with saline or FTY720 (p.o. 1 mg/kg/day).

Human Tissue Samples from Patients with Breast Cancer

Breast cancer tissue samples were collected from 7 patients who had invasive tumors larger than 1.5 cm and underwent surgical resection in Niigata University Medical and Dental

Hospital. This study protocol was approved by the Institutional Review Board of Niigata University Medical and Dental Hospital, and informed consent was obtained from all the patients. Cancerous tissue, peri-tumor normal breast tissue and normal breast tissue distant from the cancer were collected from surgical specimens immediately after operation, excised and frozen in liquid nitrogen. Peri-tumor normal breast tissue was defined as tissue within 1 cm from the gross edge of tumor, and distant from tumor was defined as tissue more than 2 cm from the gross edge of tumor. All tissue samples were stored at −80 °C.

Construction of the IF Collection Tube

Based on the previous "nylon basket" approach to the collection of IF developed by Wiig et al., [3, 18, 19] an IF collection tube was constructed by gluing Spectrum/Mesh nylon filters (20 μM mesh, 55 μM thick, Spectrum Labs. Inc., Rancho Domingguez, CA) to the bottom of Wizard Minicolumn Inserts (Promega, Madison, WI) after removing the original bottom filter. These were then placed on top of the spin columns (Fig. 1).

Collection of IF from Tissues

Animals were sacrificed by exsanguination, blood was collected, and tissues were harvested for IF collection by an established method [3, 18, 19] with some modifications. Briefly, tissue was excised, blotted gently, and placed in pre-weighed tubes on ice. Tubes were re-weighed to determine tissue weight and the tissue was sectioned several times with scissors. The samples were then transferred into the inserts with nylon mesh, and placed into the pre-weighed centrifuge tubes. The tubes were centrifuged at 106 × g for 10 min at 4 °C and the IF accumulated below the filter. The volume of IF was quantified by weight. PBS containing phosphatase inhibitors (100 μl) was added to the IF and the tubes were centrifuged at 1000 × g for 10 min at 4 °C to remove any contaminating cells (Fig. 1).

Cell Counting and Immunoblot

Cell numbers were determined in IF without centrifugation, after centrifugation at 106 × g, 1000 × g, 10,000 × g by counting the numbers under microscopy. Protein from lymph node (LN) tissue extracts or from IF were quantified by western blotting with anti-actin antibody and stained with Ponceau S to visualize proteins. Densitometry of the blot was assessed using Image J software, and the relative level of actin was normalized with equal protein amount of LN and IF.

Quantitation of Sphingolipids by LC-ESI-MS/MS

Lipids were extracted from IF, blood, or tissue samples and sphingolipids were quantified by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC-ESIMS/MS, 4000 QTRAP, ABI) as described previously [12, 20, 21].

Statistical Analysis

Results were analyzed for statistical significance with a two-tailed Student's t-test, with $P < 0.05$ considered significant. Experiments were repeated at least three times with consistent results.

Results

An Improved Method to Collect IF from Tissues

Although it has been suggested that S1P levels are relatively low in IF compared to cells, this has not been verified experimentally [22]. We modified an established method by Wiig et al. [3, 18, 19] and developed a new IF collection tube to enable efficient collection of IF from small tissue samples for sphingolipid measurements (Fig. 1). The recovery of IF was low from tissues weighing less than 200 mg, while the volume of IF collected was proportional to the weight of tissues weighing more than 200 mg (Fig. 2a). To protect S1P from degradation, buffer containing phosphatase inhibitors was added to the IF, and a subsequent centrifugation at 1000 × g was used to remove contaminating cells (Fig. 2b). To examine whether collected IF contained cells or components of broken cells, 10 μg of protein in IF from lymph node tissue and the same amount of protein extracted from lymph node tissue were separated by SDS-PAGE and immunoblotted with an antibody to actin, the major intracellular protein. Actin was barely detectable in the IF (Fig. 2c). Densitometric analysis of the actin band revealed that the IF contained less than 0.3 % of the actin protein as compared to the same amount of protein extracted from lymph node tissue (Fig. 2c). Repeated analyses of IF samples demonstrated minimal variation (i.e. tight error bars), also indicating low contamination.

Effect of Deletion of SphK1 or SphK2 on Sphingolipid Levels in Blood

As we have an interest in investigating the level and function of S1P in the various fluid compartments of the body, we initially investigated the different contributions of SphK1 and SphK2 to S1P levels in whole blood and serum using knockout mice. In agreement with previous reports [15, 23–25], levels of S1P and dihydro-S1P (DHS1P) in blood as well as in serum of SphK1$^{-/-}$ mice were lower than those

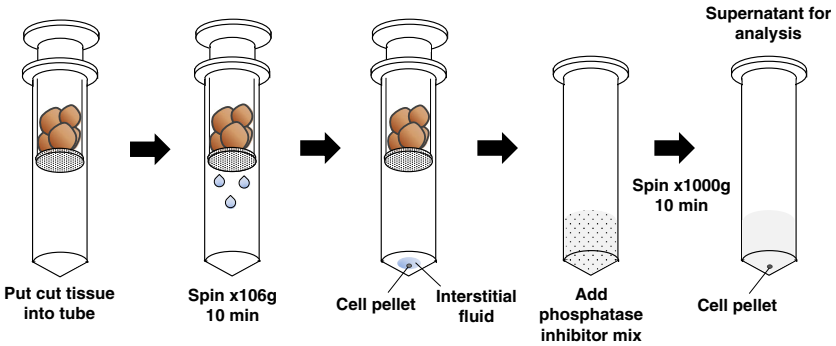

Fig. 1 Scheme for the isolation of IF from tissues. Excised tissue was placed inside pre-weighed inner tubes with nylon mesh. The tubes were centrifuged at 106 × g for 10 min at 4 °C allowing IF accumulation in the bottom of the tube together with a very small cell pellet. After weighing to determine the volume of IF, phosphatase inhibitor mix was added and the tubes were centrifuged at 1000 × g for 10 min at 4 °C to remove any contaminating cells

found in wild type (WT) littermates (Fig. 3a and 3c). In contrast, and in agreement with others [14, 26], S1P and DHS1P levels in blood as well as serum of SphK2$^{-/-}$ mice were higher than those of their WT littermates, most likely due to compensatory up-regulation of SphK1 that produces S1P and DHS1P in the SphK2$^{-/-}$ animals [14] (Fig. 3b and 3d). Hence, SphK1, rather than SphK2, appears to contribute to the S1P levels in whole blood and serum. Levels of sphingosine (Sph) and dihydro-Sph (DHSph) in blood and serum are much lower than the phosphorylated sphingoid bases in both knockouts and WT mice, as were previously reported [21, 26–29].

S1P Levels Are Higher in Mammary Gland IF than in Mammary Gland Itself

Next, it was of interest to examine the contribution of SphK1 and SphK2 to levels of bioactive sphingolipids in IF compared to the tissue it was collected from. We examined their levels in IF from mammary gland compared to the tissue itself. S1P and DHS1P levels in mammary glands were much lower than those of Sph and DHSph and there were no major differences in S1P levels between the SphK1 knockouts and their littermate controls (Fig. 4a), yet there were significant decreases in levels of DHS1P and Sph in SphK1$^{-/-}$ mice. S1P levels were slightly increased in SphK2 knockout mice compared to their littermate controls, while levels of Sph were not changed (Fig. 4b). Importantly, substantial concentrations of S1P and DHS1P were found in IF from mammary glands, which were approximately 10-fold higher than those in the tissue. Moreover, deletion of SphK1 greatly reduced levels of both phosphorylated sphingoid bases as well as Sph and DHSph (Fig. 4c). In contrast, deletion of SphK2 did not affect their levels in IF significantly (Fig. 4d).

Levels of Bioactive Sphingolipids in Breast Tumor IF Correlate with Tumor Growth

We previously showed in a syngeneic mouse breast cancer model in which 4T1-luc2 murine mammary cancer cells were orthotopically implanted into the chest mammary glands of

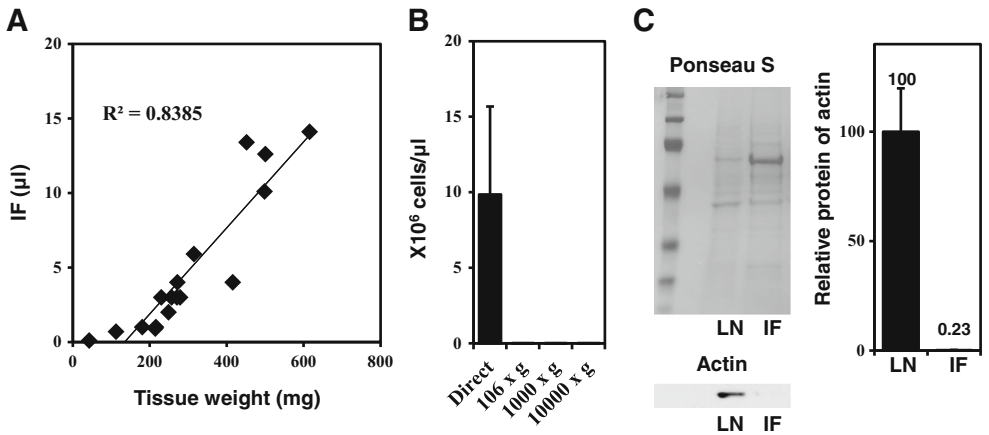

Fig. 2 Collection of interstitial fluid (IF) from tissues. **a** IF was collected from different amounts of 4T1 breast tumor tissue. Correlation between the tumor tissue weight and the volume of IF collected is shown. **b** Removal of contaminating cells from IF. Cell numbers in IF were determined after centrifugation as indicated. **c** 10 µg of protein from lymph node tissue extracts (LN) or from IF were analyzed by western blotting with anti-actin antibody (lower panel) and stained with Ponceau S to visualize proteins (upper panel). Densitometry of the blot was assessed using Image J software, and the relative level of actin was normalized with equal protein amount of LN and IF

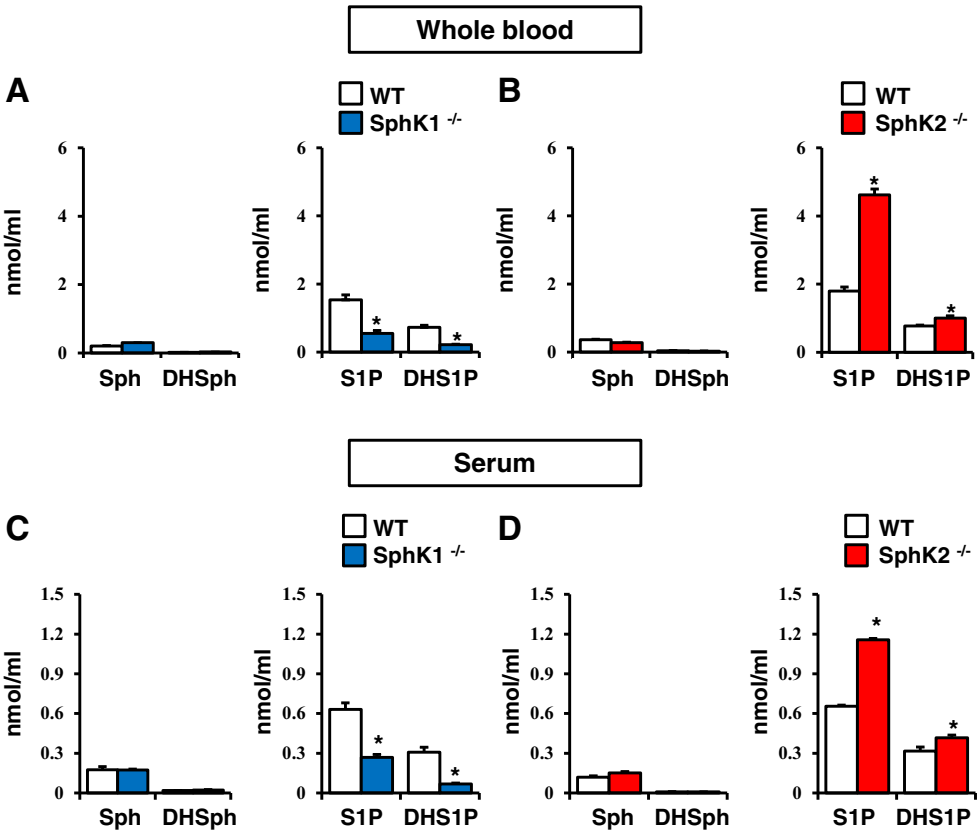

Fig. 3 Levels of sphingolipids in blood and serum from SphK1$^{-/-}$ and SphK2$^{-/-}$ mice and littermate wild type mice. Levels of sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in blood (**a, b**), and serum (**c, d**) from 2 month old SphK1$^{-/-}$ mice (*blue bars*), SphK2$^{-/-}$ (*red bars*), and their respective WT littermates (*white bars*) were determined by LC-ESI-MS/MS. Mean ± SEM ($n = 4$). *, $P < 0.05$

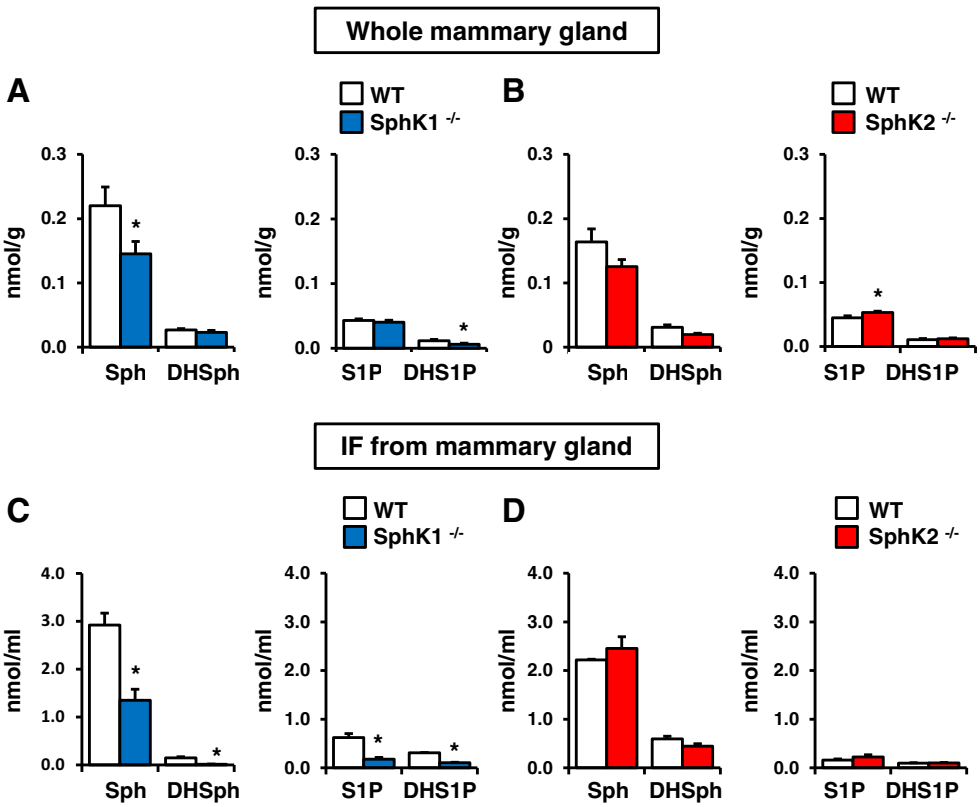

Fig. 4 Levels of bioactive sphingolipid metabolites in normal mammary glands and mammary gland IF from SphK1$^{-/-}$ and SphK2$^{-/-}$ mice and corresponding littermate wild type (WT) mice. Sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in mammary glands (**a, b**), and IF from the mammary glands (**c, d**) from SphK1$^{-/-}$ mice and WT littermates (**c**), SphK2$^{-/-}$ mice and their WT littermates (**d**) were determined by LC-ESI-MS/MS. Data are mean ± SEM ($n = 4$). *, $P < 0.05$

immunocompetent mice that S1P levels are increased in tumors and correlated with tumor growth [13]. Because we [14] and others [30, 31] have shown that oral administration of FTY720 reduces tumorigenesis, and because of the known effects of FTY720 on S1P signaling, we treated 4T1 tumor bearing mice with FTY720 and examined correlations between tumor burden and levels of bioactive sphingolipids in tumor IF. FTY720 greatly reduced tumor growth, as demonstrated by in vivo bioluminescence and tumor volume measurements (Fig. 5a–c), levels of S1P and DHS1P in tumor IF were significantly decreased compared to saline treated animals (Fig. 5d).

S1P Levels Are Higher in Breast Cancer IF than in Normal Breast Tissue IF from Human Patients

Next, we examined the levels of sphingolipids in IF from human patients with breast cancer to examine whether the observation seen in animal models is also applicable to the human patients. For this purpose, we obtained IF from breast tumor tissue and normal breast tissue from two different areas (peri-tumoral area and distant area from the tumor) in each patient with breast cancer and determined levels of sphingolipids in the fluid. Importantly, Sph, DHSph, and S1P levels, but not DHS1P, were significantly higher in the breast tumor tissue IF than in the normal breast tissue IF (Fig. 6). There is no significant difference in levels of Sph, DHSph, S1P or DHS1P between IF from normal breast tissue that is distant from tumor and that from peri-tumor normal breast tissue (Fig. 6).

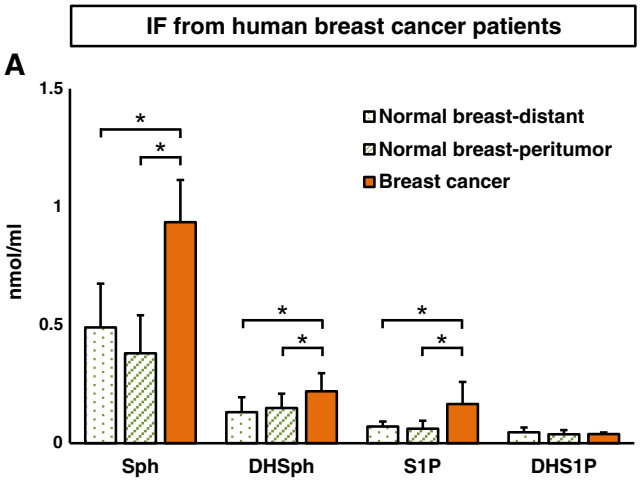

Fig. 6 Levels of bioactive sphingolipids in IF from breast tumor and normal breast tissue of patients with breast cancer. **a** IF was obtained from breast cancer tissue and normal breast tissue obtained from distant area from tumor and peritumor area. Levels of sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in the IF from tumor and normal breast tissue were determined by mass spectrometry. Data are mean ± SEM. *, $P < 0.05$

Discussion

High levels of S1P in blood are critical for maintenance of the tone and integrity of the vascular endothelium. The S1P gradient between high levels in the circulation and the low levels in tissues due to the presence of S1P degrading activity from phosphatases and S1P-lyase is important for immune cell trafficking [22]. It has been generally assumed that S1P levels in IF of lymphoid tissues are very low so that S1PR1 on lymphocytes can sense the S1P gradient as they exit into the blood. Previous studies have suggested that S1P secreted by tumor cells plays an important role in tumor progression and metastasis [13]. However, there are no reports on S1P in IF, due in part to the difficulties in collection. To our knowledge, this is the first report of the measurement of S1P and DHS1P in tumor IF in murine normal mammary glands and mammary tumors, as well as in human patients with breast cancer.

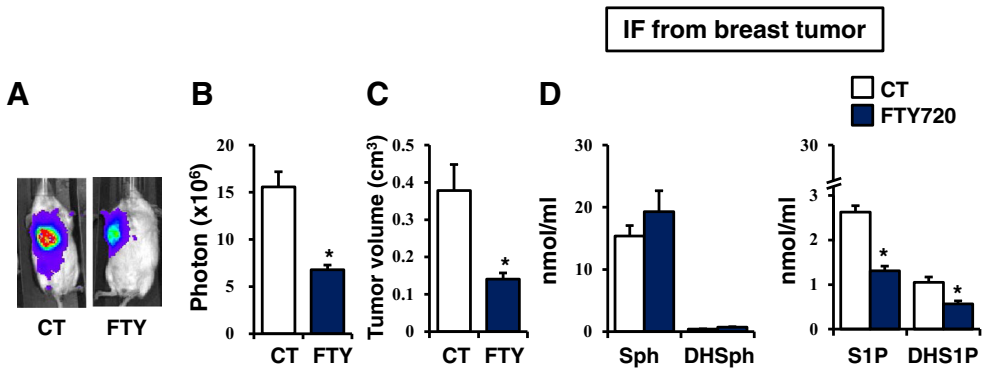

Fig. 5 Levels of bioactive sphingolipids in breast tumor IF correlate with tumor growth. 4T1-luc2 cells were surgically implanted in mammary glands. Tumor-bearing mice were randomized into 2 groups and treated daily by gavage with saline (*open bars*) or FTY720 (1 mg/kg, *black bars*). **a** Representative IVIS images of 4T1 breast tumors on day 14 after implantation. **b** Tumor burden determined by in vivo bioluminescence. Data are means ± SEM ($n = 5$). *, $P < 0.05$. **c** Tumor size was determined and volume calculated with the cylinder formula. Data are means ± SEM. *, $P < 0.05$. **d** Levels of sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in 4T1 breast tumor IF from saline or FTY720 treated mice were determined by mass spectrometry. Data are mean ± SEM. *, $P < 0.05$

Wiig et al. previously described a method to collect IF without causing cellular damage using low speed centrifugation of tissues on nylon mesh which required a large amount of tissue [3, 18, 19]. In order to collect IF from a smaller amount of tissue, we improved this method by designing a more efficient IF collection tube with a smaller nylon mesh surface area, thereby reducing loss of IF from absorption of the fluid by the mesh. Based on our experience, we recommend the use of at least 400 mg tissue for reproducible collection of IF. Nevertheless, LC-ESI-MS/MS is sufficiently sensitive to accurately measure sphingolipids in a volume of IF of less than 10 μl. As was reported by Wiig et al. [3, 18], we also found negligible contamination of IF with cellular components from contaminating or broken cells as shown by the extremely low amounts of actin.

Using the simple method for collection of IF that we have described, we have been able to validate previous assumptions regarding extracellular S1P as well as discover several new insights into the role of S1P in the tumor microenvironment. Levels of S1P in normal mammary glands are known to be relatively low, much lower than Sph; however, we have found high concentrations of bioactive sphingolipids (reaching 0.6 μM S1P and 0.2 μM DHS1P) to be present in IF from normal mammary glands. Sphingolipid metabolites in mammary gland IF from SphK1$^{-/-}$ mice were significantly decreased, suggesting that it is SphK1 that plays a pivotal role in regulating levels of these metabolites in IF from normal mammary glands. Though in vitro studies have suggested that it is SphK1 and not SphK2 that is the major contributor of secreted S1P, this is the first study to validate this in an in vivo setting.

FTY720 is a pro-drug approved for treatment of multiple sclerosis. It is phosphorylated in vivo to FTY720-phosphate, a S1P mimetic that modulates S1PR functions [32]. However, we [14] and others [30, 31] have shown that FTY720 also potent anti-cancer activities. In agreement, we found that oral administration of FTY720 greatly reduced breast tumor growth in a syngeneic model. Importantly, S1P and DHS1P levels in tumor IF were significantly decreased by FTY720 administration and correlated with the reduction of tumor growth. While this observation further supports the notion that S1P may have an important role within the tumor microenvironment, it also provides an important insight into the possible mechanisms of action of FTY720 on cancer progression. Though FTY720 in its phosphorylated form is known to have its immunosuppressive effects as a functional antagonist of S1PR1, inducing internalization and degradation of S1PR1 and prolonged receptor downregulation, it has also been shown that FTY720 inhibits SphK1 and induces its proteasomal degradation [33, 34]; therefore, the lower levels of S1P in the tumor IF from tumor bearing mice treated with FTY720 compared to saline treated animals could also be due to inhibition or reduction of SphK1 in the breast cancer cells. SphK1 is known to be upregulated in many cancers including breast [35–39] and we have shown that tumor bearing mice have increased systemic S1P [13] and may communicate with the host via the systemic SphK1/S1P axis to regulate lung metastasis/colonization [40]. Our findings suggest the possibility that S1P secreted from tumor cells to IF may be important for metastasis by stimulating S1P signaling important for cancer progression and highlights its important role in the tumor microenvironment. Further studies to investigate the roles of tumor IF in cancer progression is necessary to address this issue.

Acknowledgments We gratefully acknowledge the VCU Lipidomics Core, which is supported in part by funding from the NIH-NCI Cancer Center Support Grant P30CA016059.

Author Contribution MN and KT conceived the study. MN, AY, TA, WCH, KPT, BA, carried out experiments. HM developed the IF collection tube. JCA performed mass spectrometry analysis. MN and TK wrote the manuscript with assistance from OMR, TW, SS and SM.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

Funding MN was a Japan Society for the Promotion of Science Postdoctoral Fellow. MN is supported by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research Grant Number 15H05676 and 15 K15471, the Uehara Memorial Foundation, Nakayama Cancer Research Institute, Takeda Science Foundation, and Tsukada Medical Foundation. JT is supported by the JSPS Grant-in-Aid for Scientific Research Grant Number 16 K19888. KT is supported by NIH/NCI grant R01CA160688 and Susan G. Komen Investigator Initiated Research Grant IIR12222224. SS is supported by NIH/NCI grant R01CA61774 and in part by the DoD BCRP program under award number W81XWH-14-1-0086. KPT is supported by T32CA085159.

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Modified breast cancer model for preclinical immunotherapy studies

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ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form
13 April 2016
Accepted 1 June 2016
Available online 8 June 2016

Keywords:
Breast cancer
Syngeneic model
Preclinical
Immunotherapy

ABSTRACT

Background: Interest in immunotherapy for breast cancer is rapidly emerging, and applicable animal models that mimic human cancer are urgently needed for preclinical studies. This study aimed to improve a technique for orthotopic inoculation of syngeneic breast cancer cells to be used as a preclinical animal model for immunotherapy.

Materials and methods: We used our previously reported murine model of orthotopic cancer cell inoculation under direct vision and compared the efficiency of tumorigenesis with tumor cells suspended in either phosphate-buffered saline or Matrigel containing varying numbers of cells. As a model for immune rejection, murine BALB/c–derived 4T1-*luc2* breast cancer cells were inoculated orthotopically into both BALB/c and C57BL/6 mice.

Results: Matrigel-suspended cells formed larger tumors with higher efficiency than phosphate-buffered saline-suspended cells. The maximum volume of Matrigel that could be inoculated without spillage was 20 μL and 30 μL in the #2 and #4 mammary fat pads, respectively. Tumor take rates increased as the injected cell number increased. In this immune rejection model, there were no significant differences in tumor weight between the strains up to day 7, after which tumor weight decreased in C57BL/6 mice. Bioluminescence in C57BL/6 mice was also significantly less than that in BALB/c mice and increased up to day 7, then swiftly decreased thereafter.

Conclusions: This improved technique of innoculating murine breast cancer cells using bioluminescence technology may be useful in evaluating the efficacy of tumor regression mediated by immune responses, as shown by an allogeneic response in C57BL/6 mice.

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http://dx.doi.org/10.1016/j.jss.2016.06.003

Introduction

Immunotherapy, including checkpoint inhibitors, cancer vaccines, adoptive cell immunotherapy, and strategies that exploit chimeric antigen receptor–engineered T cells, is rapidly emerging as a promising modality for different types of cancers.[1] The early success of immune checkpoint inhibition, such as targeted therapy against cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), along with programmed death-1 (PD-1)[2,3] and the PD-1 ligand, PD-L1,[4] has drawn much attention.[5]

One of the most commonly used animal models for oncologic preclinical studies is xenografts of human cancer cells or patient-derived cancer tissue into immune-deficient mice.[6,7] However, these models are not suitable to evaluate immune responses or the effects of immunotherapy because the host animal lacks a fully functioning immune system. Spontaneous tumorigenic models using transgenic mice or carcinogens have been developed in animals with an intact immune system, but these models may require a long waiting time for the development of cancer, which limits their practicality.[8,9] Furthermore, these models usually require expensive equipment, such as mouse-specific imaging scanners (computed tomography, magnetic resonance imaging, or positron emission tomography), to detect and measure tumors. Even with such state-of-the-art diagnostic imaging equipment, the evaluation of immunotherapy responses remains challenging. Tumor size may not necessarily reflect the amount of cancer cells, as tumors may initially enlarge on imaging studies due to accumulated infiltrating immune cells when immunotherapy is actually effective. Recently, patient-derived "humanized" xenograft models have been developed using patient tumors implanted into immune-deficient mice that are engineered to have intact human immune cells.[10-12] However, the cost of these animals and other limitations, including take rate, viral contamination and selection pressure, hinder this approach,[10] and even these modern models cannot escape from the challenges of assessing tumor responses to immunotherapy.[12] Thus, orthotopic inoculation of syngeneic murine tumor cells tagged with a bioluminescent reporter into immune intact mice is the most straightforward, fast, and affordable model to study the effect of immunotherapy at this point.

We have previously reported the establishment of a murine syngeneic breast cancer model using cell inoculation into chest mammary fat pads under direct vision, which can mimic human cancer progression.[13-15] In this study, we report the establishment of an improved orthotopic inoculation technique of murine breast cancer cells using *luciferase*-tagged 4T1-*luc2* murine cancer cells suspended in Matrigel and demonstrate that this model is useful to assess immune-mediated regression of breast tumors.

Materials and methods

Cell culture

4T1-*luc2* cells, a mouse mammary adenocarcinoma cell line derived from BALB/c mice that has been engineered to express luciferase was purchased from Caliper Life Sciences/PerkinElmer (Hopkinton, MA). 4T1-*luc2* cells were cultured in RPMI Medium 1640 with 10% fetal bovine serum. E0771 cells, a C57BL/6 mouse mammary fat pad–derived adenocarcinoma cell line, were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum.

Animal models

Approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee was obtained for all experiments. Female BALB/c and C57BL/6 mice were obtained from Jackson Laboratory. All cell inoculations into #2 (chest) and #4 (abdomen) mammary fat pads under direct vision were carried out as previously described.[13] All procedures were performed using sterile technique under isoflurane anesthesia, and the animals were prepped and draped in a sterile manner. In brief, a 10-mm incision was made medial to the nipple, and a cotton swab was used to expose the mammary gland. A syringe with a 26-G needle was used to inject the cell suspension directly into the mammary gland under direct vision, and the wound was closed with a suture. To maximize the take rate, cell inoculations were conducted within 1 h from preparation of cell suspensions. Tumor growth was monitored by caliper measurement, and animals were weighed every other day.

Preparation of Matrigel and phosphate-buffered saline cell suspensions

1×10^4 of 4T1-*luc2* cells were suspended in 20 μL of either phosphate-buffered saline (PBS) or Matrigel. Cells were inoculated into #2 and #4 mammary fat pads of BALB/c mice ($n = 8$). Fourteen days after inoculation, tumors were assessed by palpation, then harvested, and weighed.

Determination of the optimum amount of Matrigel for injection into mammary fat pads

To determine the amount of Matrigel a mammary fat pad can hold, 10 μL of Matrigel stained with 10% Trypan blue was injected incrementally into the #2 and #4 mammary fat pads ($n = 12$, each group). Spillage of Matrigel out of the fat pad was assessed visually after each injection.

Tumorigenesis with different numbers of cells inoculated

4T1-luc2 cells (5×10^2, 1×10^3, 5×10^3, 1×10^4) or E0771 cells (5×10^4, 1×10^5, 5×10^5, 1×10^6) were suspended in 20 μL of Matrigel, then inoculated into the #2 and #4 mammary fat pads of C57BL/6 or BALB/c mice, respectively ($n = 8$, each group). Formation of tumors was determined by palpation. Larger numbers of E0771 cells were inoculated because of the slower growth rate of this tumor in the appropriate syngeneic mouse strain.

4T1-luc2 tumors in C57BL/6 mice (immune rejection model)

1×10^4 of 4T1-*luc2* cells, derived from BALB/c mice, suspended in Matrigel were implanted into the right #2 fat pad of C57BL/6 mice or BALB/c mice as a control. Tumor growth was monitored every other day by bioluminescence (IVIS)

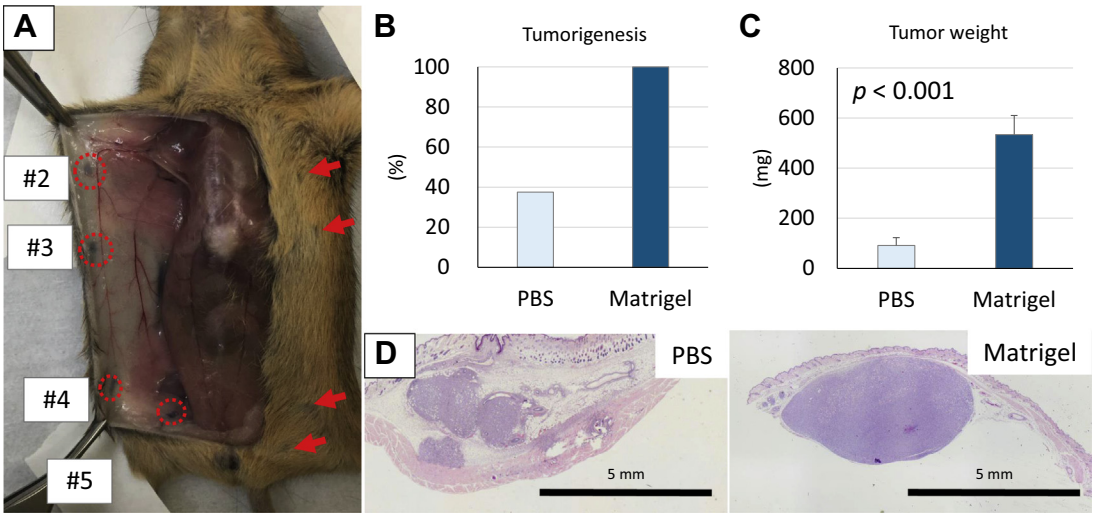

Fig. 1 – Location of mouse mammary fat pads and the difference in tumorigenesis of cancer cells suspended in Matrigel or PBS. (A) The locations of mouse mammary fat pads (#2-5) were identified after midline incision (red circles and arrows). This representative image depicts the ease to access #2 and #4 mammary fat pads for inoculation under direct vision. Note, #1 pads cannot be seen, and #3 pads are too small, and #5 pads are too deep at the base of the legs for accurate inoculation. (B) Tumorigenesis 2 wk after inoculation of 1×10^4 4T1-*luc2* cells. (C) Tumor weights were significantly greater in the tumors from Matrigel-suspended cells, than from the PBS-suspended cells ($n = 8$). (D) Histologic examination showed that inoculation of PBS-suspended cells resulted in scattering of cancer cells in the mammary fat pad, and cancer cells were also seen in secondary tumors outside the mammary fat pad, whereas Matrigel-suspended cells formed single tumors confined to the mammary fat pad. (Color version of figure is available online.)

imaging ($n = 4$). For tumor weight analysis, cells were inoculated into bilateral #2 and #4 fat pad, and tumors were harvested and weighed on days 3, 7, and 9 ($n = 8$, each). Pathologic analyses were performed after formalin fixation of the tumors. Tumor sections were prepared and stained with hematoxylin and eosin by Virginia Commonwealth University Health System Anatomic Pathology Research Services.

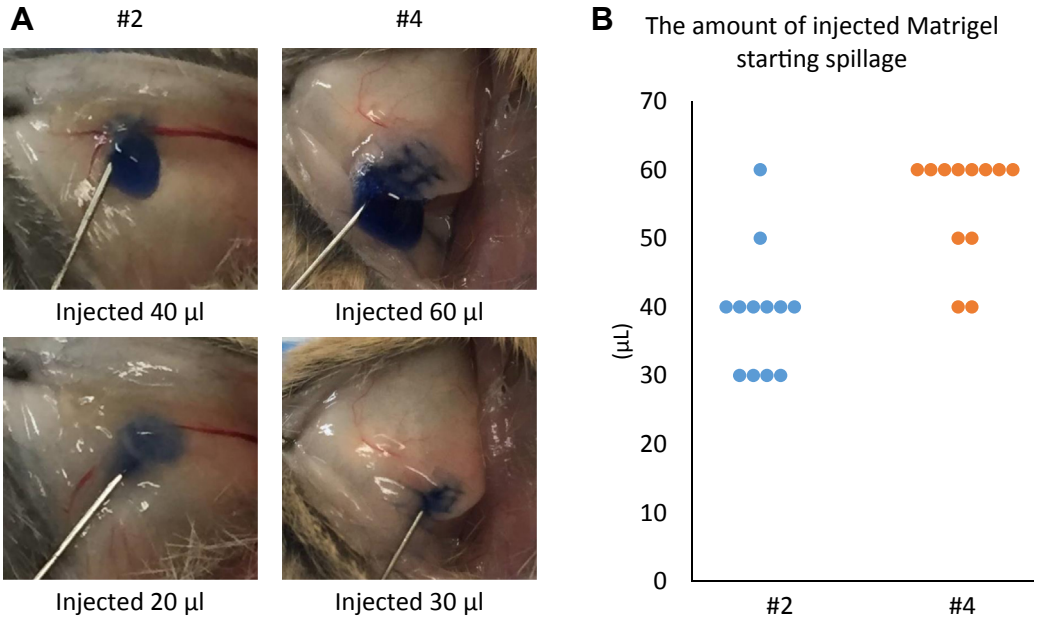

Fig. 2 – The amount of Matrigel a mammary fat pad can hold without spillage. Trypan blue–stained Matrigel was injected into mammary fat pads in 10-µL increments, and spillage was detected visually. (A) Representative images of Trypan blue–stained Matrigel inoculation. Spillage out of the mammary fat pads is observed in the 20-µL injection in #2 (left) and 30-µL injection in #4 (right) mammary fat pads. (B) The amount of Matrigel that began to spill out of the pads when inoculated to either #2 or #4 mammary fat pads. (Color version of figure is available online.)

Bioluminescent quantification of tumor burden

Xenogen IVIS-200 and Living Image software (Caliper Life Sciences/PerkinElmer) were used to quantify the photons per second emitted by 4T1-*luc2* cells. D-Luciferin (150 mg/kg; PerkinElmer) was injected intraperitoneally into mice previously implanted with 4T1-*luc2* cells. Bioluminescence was measured and quantified at 5-min intervals up to photocount peak. Bioluminescence was then determined by the peak number of photons per second calculated over this time frame.[15]

Statistical analyses

Statistical analyses were performed by the chi-square test or Fisher exact test with a single degree of freedom, and the Student's *t*-test was used to analyze the differences between two groups. *P* values <0.05 were considered to have statistical significance. All statistical analyses were performed using SPSS version 23.0 (SPSS, Chicago, IL).

Results

Comparison of tumorigenesis after inoculation of cell suspensions in Matrigel or PBS

The locations of mouse mammary fat pads are illustrated in Figure 1A after injection of 10% Trypan blue dye through the skin close to the nipple. The image shows the relative ease of access to #2 and #4 mammary fat pads for inoculation under direct vision from exposure through a midline incision. Notable in Figure 1A, #1 pads are not visible despite wide exposure, #3 pads are too small to access, and #5 pads are too deep at the base of the legs for accurate inoculation. Therefore, #2 and #4 mammary fat pads were used for orthotopic inoculation of breast cancer cells in subsequent experiments.

In our previous study, we found that inoculation of cell suspensions in PBS resulted in spillage out of the mammary fat pads, with scatter of cells outside the pads.[13] Therefore, the volume injected was minimized, which limited the number of cells that could be inoculated. We now have compared the efficiency of generating 4T1-*luc2* tumors from cells suspended in Matrigel with that of PBS-suspended cells in our orthotopic inoculation under direct vision model. Only 3 of 8 mice (37.5%) developed palpable tumors from cells injected in PBS, whereas all mice (8 of 8, 100%) developed tumors after inoculation of tumor cells suspended in Matrigel by 2 wk (Fig. 1B). Tumors generated from Matrigel-suspended cells weighed significantly more than those generated from cells suspended in PBS ($P < 0.001$; Fig. 1C). In agreement with our previous observations,[13] inoculation of PBS-suspended cells resulted in cancer cells forming tumors not only in the mammary fat pad but also in subcutaneous tissue outside of the mammary fat pad (Fig. 1D left panel) In contrast, Matrigel-suspended cells formed single tumors confined to the mammary fat pad (Fig. 1D right panel). These results demonstrate that cells suspended in Matrigel are more efficient at generating a discrete primary breast tumor in our orthotopic model.

Determination of the amount of Matrigel a mammary fat pad can hold

We then investigated the amount of Matrigel that the #2 or #4 mammary fat pads can hold without spillage. The amount of injectate (Trypan blue–stained Matrigel) was incrementally increased by 10 μL until spillage occurred. As shown in Figure 2A and B, spillage was observed when more than 20 μL or 30 μL of Matrigel was injected into the #2 or #4 mammary fat pads, respectively. Therefore, 20 μL and 30 μL of Matrigel were identified to be the most suitable amounts to inject into #2 or #4 fat pads, respectively.

Dependence of tumor formation on number of cells inoculated

To examine the relationship between inoculated cell number and generation of tumors, increasing numbers of cells were inoculated into the #2 or #4 mammary fat pads and tumorigenesis monitored. We used the two most commonly used syngeneic breast cancer models, 4T1-luc2 cells into BALB/c

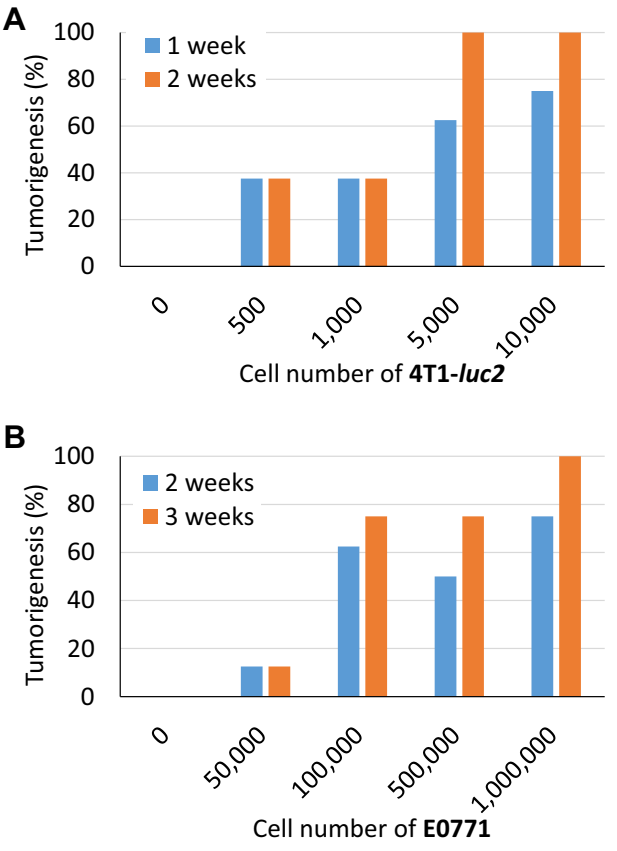

Fig. 3 – Number of cells in Matrigel inoculates required to generate palpable tumors in two syngeneic breast cancer models. Shown on the X axes are the numbers of 4T1-luc2 cells inoculated into mammary fat pads of BALB/c mice ($n = 8$; A) and E0771 cells inoculated into C57BL/6 mice ($n = 8$; B). The proportion of mice with palpable tumors (tumorigenesis) was assessed at 1 and 2 wk after inoculation for 4T1-luc2 and 2 and 3 weeks for E0771. (Color version of figure is available online.)

mice and E0771 cells into C57BL/6 mice. We found that tumorigenesis increased as the inoculated cell number increased for both cell lines, where all animals developed a tumor with 5×10^3 4T1-luc2 cells, and 1×10^6 E0771 cells (Fig. 3A and B).

Tumor growth in different background mice

To investigate whether our model is appropriate to assess immune-mediated regression of breast tumors, we examined the growth of 4T1-*luc2* cells, inoculated in Matrigel, in an environment where the cells will be rejected by an allogeneic immune response. 4T1-*luc2* cells were orthotopically inoculated into either syngeneic (BALB/c) or allogeneic (C57BL/6) mice. Thus, the tumors growing in C57BL/6 mice would be expected to be destroyed by the native immune system. The number of viable cancer cells and tumor burden were monitored *in vivo* by bioluminescence (Fig. 4A). Two-fold increases in tumor growth measured by bioluminescence imaging were nearly identical in both backgrounds 24 h after inoculation. By 7 d after inoculation, 4T1-*luc2* cells in C57BL/6 mice grew approximately 160 fold their inoculated amount, whereas the tumors in BALB/c mice grew 1200 fold their inoculated amount (Fig. 4B). The BALB/c tumors continued to grow rapidly to reach an almost 10,000-fold increase in bioluminescence signal, whereas the C57BL/6 mice tumors swiftly decreased the signal from day 7, and the viable cancer cells were eliminated by day 14. We further analyzed the time after injection of luciferin required to reach a peak photocount, which is

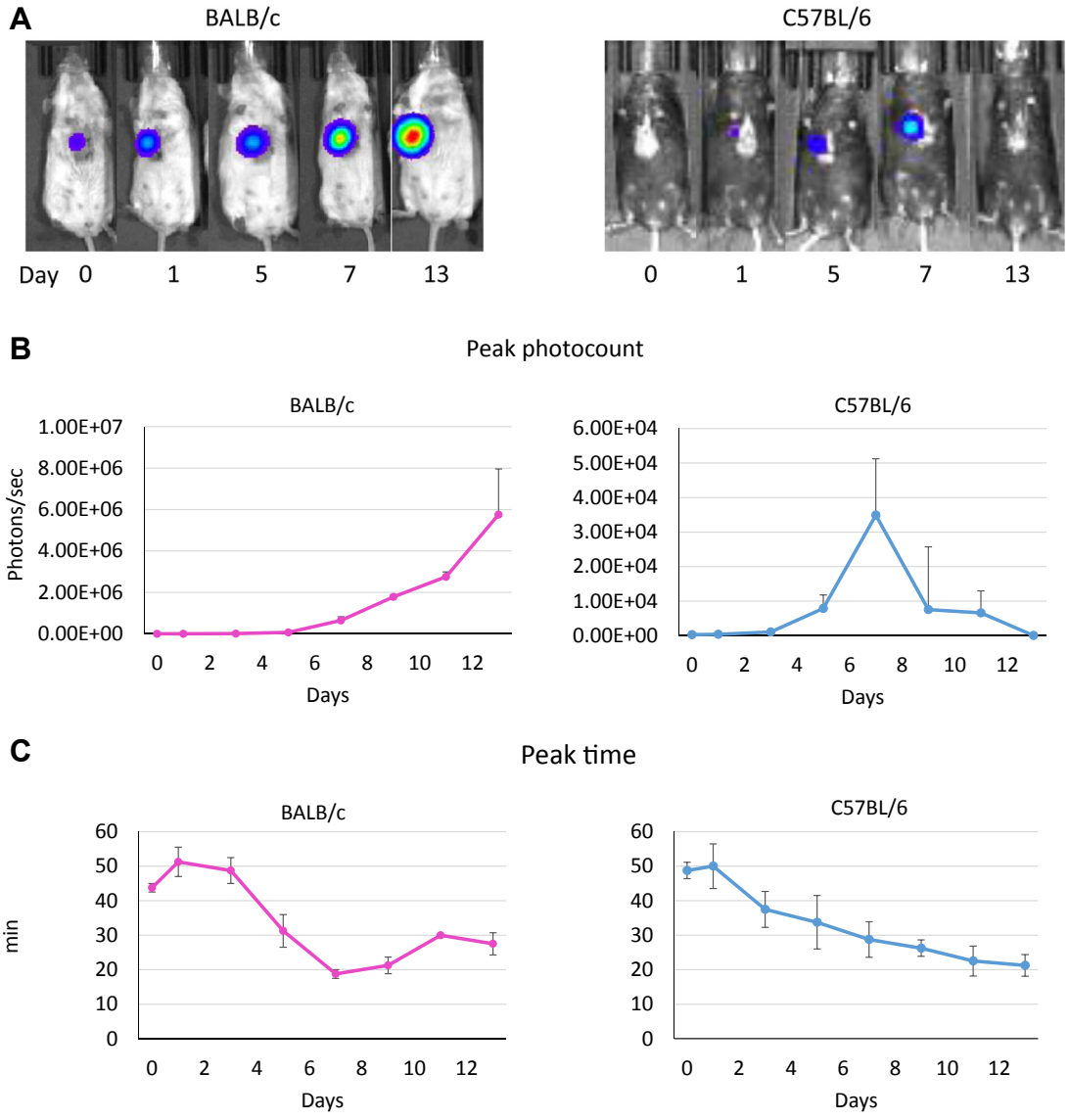

Fig. 4 – 4T1-*luc2* breast cancer growth after orthotopic inoculation into the mammary pads of BALB/c or C57BL/6 mice. BALB/c or C57BL/6 mice were orthotopically inoculated with 1×10^4 4T1-luc2 cells under direct vision, and tumor growth was evaluated by bioluminescence every other day. (A) Representative bioluminescence images of 4T1-*luc2* tumors after inoculation in BALB/c or C57BL/6 mice that showed tumor growth during observation period. (B) Tumor burden determined by photon counts was measured every other day ($n = 4$, mean ± standard error of the mean). (C) The time required to reach a peak photocount continued to decline in C57BL/6 mice, whereas it increased by day 10 in BALB/c mice. (Color version of figure is available online.)

presumably reflective of perfusion of the tumor. Time to reach a peak photocount declined by day 7 in both BALB/c and C57BL/6 mice, after which it continued to decline in C57BL/6 tumor by day 13, whereas it increased after day 10 in BALB/c mice, most likely reflecting the growth of the tumor.

There were no significant differences in tumor weights at days 3 and 7 between tumors in BALB/c and C57BL/6, but a significant difference was observed by day 9 ($P = 0.0132$; Fig. 5A). Histologic analysis showed that cancer cells were surrounded by inflammatory cells on day 9 in the C57BL/6 mice and were destroyed by day 15 in the C57BL/6 mice, whereas cancer cells without inflammatory infiltrate filled the tumor in BALB/c mice (Fig. 5B).

Discussion

For the last 8 y, our laboratory has been studying murine cancer models that mimic human cancer progression to be used for preclinical trials.[6] Our conclusion to date is that no murine model perfectly mimics human cancer progression; thus, multiple models should be used for preclinical studies to supplement each other's weaknesses.[6,7] Given the essential role of immune cells and inflammation in cancer progression, the preclinical results obtained using immune-deficient mice can be misleading and may be contributing to the low success rate of anticancer drug development.[6] Our prior work has

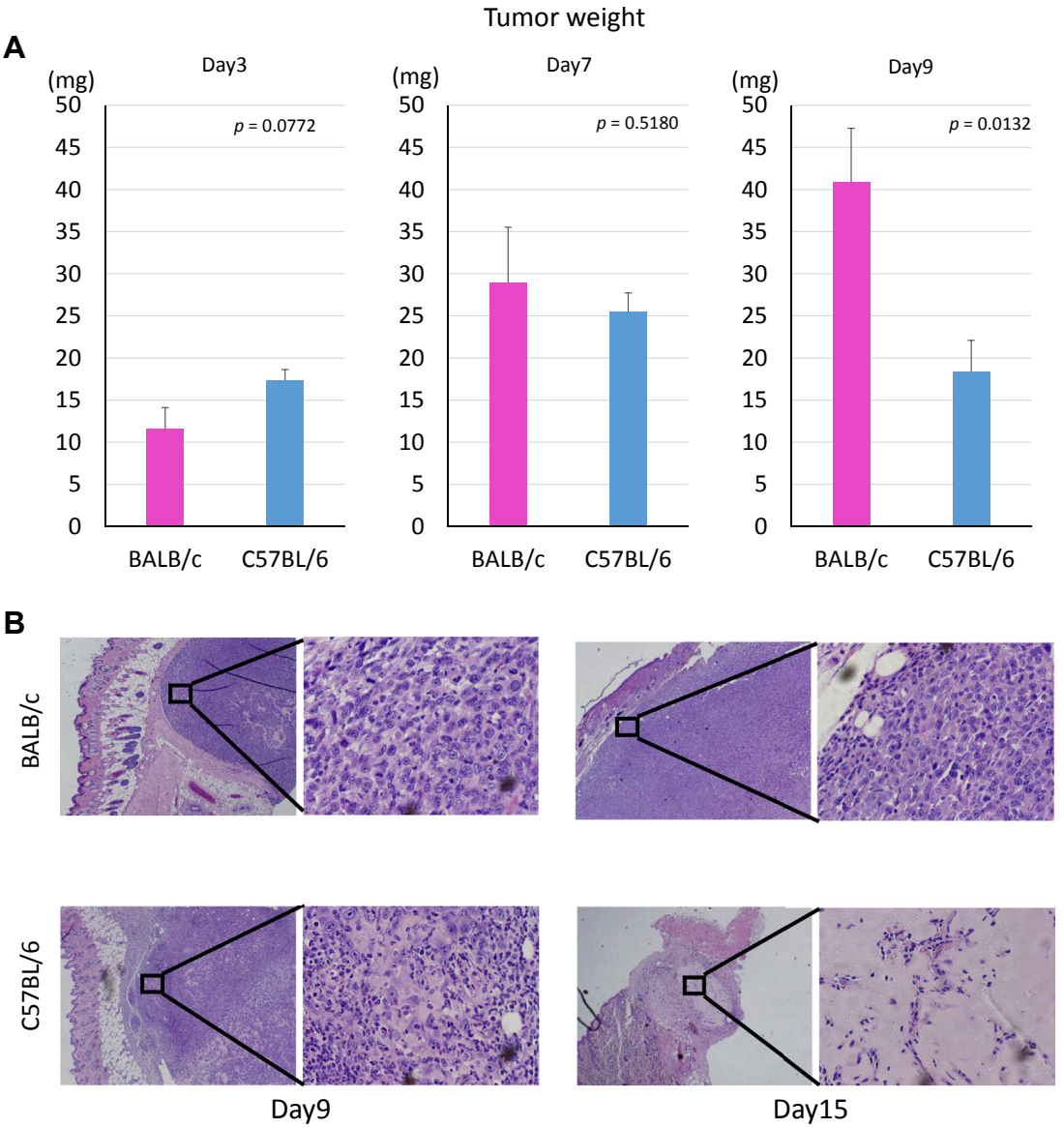

Fig. 5 – Tumor weights and histologic analyses of 4T1-*luc2* tumors in BALB/c and C57BL/6 mice. (A) Tumors were harvested and weighed on days 3, 7, and 9 ($n = 8$, each). There were no significant differences in tumor weights on days 3 and 7 between tumors in BALB/c and C57BL/6. In contrast, a significant difference was observed on day 9. (B) Hematoxylin and eosin staining of 4T1-*luc2* tumors in BALB/c and C57BL/6 mice. Cancer cells were surrounded by inflammatory cells on day 9, and cancer cells were not seen on day 15 in the tumor site of C57BL/6 mice. (Color version of figure is available online.)

shown that inoculation of syngeneic mouse cancer cells orthotopically into the mammary fat pads of immune intact mice under direct vision provides us with the most stable results that mimic human cancer progression.[13,16] This was also shown in a murine model of colon cancer, where cell suspension in Matrigel was found to be efficacious.[17] Together with previous reports that cell suspension in Matrigel facilitates the establishment of tumors in xenograft models,[18] our preliminary work led us to the present study to improve our breast cancer model using Matrigel. Compared with cells suspended in PBS, cells suspended in Matrigel developed tumors more efficiently and were more representative of primary breast tumors. We also identified the appropriate amount of Matrigel to be injected without spillage into the #2 and #4 mammary fat pads and found that the take rate correlated with the cell number inoculated. To our knowledge, this is the first to report this type of improved model using Matrigel for luciferase-tagged syngeneic breast cancer cell inoculation in the mammary fat pad under direct vision.

To test whether our improved model is suitable for preclinical evaluation of immunotherapy, we decided to investigate 4T1-*luc2* cell growth in a model invoking immune rejection, namely implantation of murine cancer cells into immune intact allogeneic mice. BALB/c-derived 4T1-*luc2* cells continued to proliferate in C57BL/6 mice for 1 wk before being eliminated, most likely reflecting the time to develop a primary immune response. Of note, the peak photon counts quantified by bioluminescence in immune-compatible BALB/c mice were approximately 12-fold and 240-fold higher compared with those in C57BL/6 mice on day 3 and day 7, respectively. Strikingly, there were no significant differences in tumor size between BALB/c and C57BL/6 mice on day 3 and day 7. Histologic analyses revealed that there were numerous infiltrating lymphocytes in the 4T1 tumors implanted in C57BL/6 mice, whereas predominantly, cancer cells were seen in BALB/c mice on day 9, suggesting that the tumor size in the C57BL/6 mice was maintained in part by tumor-infiltrating immune cells in the process of eliminating the cancer. Indeed, on day 15, no viable cancer cells were detected in the mammary pad of C57BL/6 mice. We also measured the time to reach the peak photon counts quantified by bioluminescence after intraperitoneal injection of D-luciferin, which is reflective of diffusion of the substrate into cancer cells. In agreement with a previous report,[19] the time to peak is prolonged after day 7 in BALB/c tumors after the enlargement of the tumor, whereas the time to peak continued to shorten in C57BL/6 tumor due to the tumor shrinkage.

One of the challenges of immunotherapy research is the method to evaluate the response to treatment. Commonly, the effect of systemic chemotherapy is evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) criteria, which relies on tumor size reduction.[20] In contrast with chemotherapy's immediate and direct cytotoxic and tumor shrinkage effect, immunotherapy stimulates the patient's immune system to mount an antitumor response, which includes infiltration of immune-related cells into the tumor. This often results in a paradoxical transient increase in tumor size, which can mislead the physician to perceive this as treatment failure and result in premature termination of an effective immunotherapy. This is also the case in preclinical animal studies where viable cancer cells and not the size of the tumors need to be monitored. This paradox was observed in the present study as well, when the cancer cell number monitored by bioluminescence was significantly lower in the C57BL/6 immune rejection model when compared with BALB/c model, while the measured tumor sizes were not different. This further highlights the benefits of our luciferase-tagged syngeneic cell inoculation model, which could be used to assess immunotherapy for breast cancer, such as adoptive immunotherapy, cancer vaccines, or immune checkpoint inhibitors.

Conclusions

In conclusion, we have established an improved murine orthotopic model using Matrigel, in combination with bioluminescence technology, which may be useful for preclinical studies of immunotherapy.

Acknowledgment

This work was supported by NIH grant R01CA160688 and Susan G. Komen Foundation Investigator Initiated Research grant (IIR12222224) to K.T., and Department of Defense BCRP Program Award (W81XWH-14-1-0086) to S.S.

Authors' contributions: E.K., S.C.D., and K.P.T. conceptualized and performed experiments described in the article. E.K., S.S., G.Q.P., and H.D.B. prepared the article. K.T. provided supervision of experiments and preparation of the article.

Disclosure

There are no potential conflicts of interest to disclose.

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